

INTESTINAL ANTIBODIES AND INTRA-EPITHELIAL

LYMPHOCYTES IN POTENTIAL COELIAC DISEASE

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DECLARATION

I declare that this thesis has been composed by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

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SUMMARY

The definition of coeliac disease has been reviewed in recent years. This thesis refers to the pathological and immunological abnormalities found in coeliacs and other patients with similar gastro-intestinal symptoms but a morphologically normal biopsy. Some of these abnormalities have been reported in latent coeliac disease, patients who have had a normal mucosa while eating a normal diet but have later developed a gluten-related enteropathy. The concept was first described in patients with dermatitis herpetiformis (DH), but normal intestinal mucosa, in which experiments of gluten loading led to villous atrophy.

In the early stages of the work, Dr O'Mahony showed that DH patients without enteropathy had an abnormal pattern of antibodies in jejunal fluid, similar to that found in untreated and treated coeliacs, after morphological resolution of the mucosa; and therefore, the coeliac-like intestinal antibody (CIA) pattern might be a marker of latent coeliac disease in other situations. I thus studied prospectively the frequency of this intestinal antibody pattern amongst non-coeliac patients referred for diagnostic small bowel investigations, eventually found to have a normal jejunal biopsy.

There was report of a high $\tau\delta$ intra-epithelial lymphocyte

(IEL) count in a normal biopsy of a patient years before the development of enteropathy. Thus, I set up the collection of biopsies suitable for frozen sections, and the study of IEL bearing the $\gamma\delta$ form of TCR. I thought that CIA+ patients, particularly those with a high IEL count, would also have high $\gamma\delta$ IEL counts. I assessed the possibility of relationships between the CIA pattern, a high $\gamma\delta$ IEL count and the other putative markers of latent coeliac disease, the serum anti-gliadin antibodies and an abnormal permeability test. The term potential coeliac disease was used to describe patients positive for any of these indices identified in the course of our research.

I tested the usefulness of jejunal fluids for the study of humoral intestinal immunity, as a product of the local secretion, by measuring possible leakage of plasma-derived proteins (albumin, α -1 anti-trypsin), and the proportion of IgA bound to secretory component (SC).

In order to explain the immunopathogenesis of the CIA pattern, some mechanisms were suggested: a primary abnormality of B cells, a dysfunction of T cells involved in B cell switching, or the existence of an immunostimulatory factor in mucosa; and some strategies were developed for the study of these mechanisms. I counted lamina propria plasma cells in patients positive and negative for the CIA pattern. CIA positive patients had a high number of IgM+ cells (thus up-regulation of IgM

responses), and also a high proportion of sIgA in jejunal fluid, suggesting that neither the B cell population, nor the epithelial transport of these immunoglobulins are affected, but rather the immuno-regulatory factors controlling these processes.

The CIA pattern might reflect a low-grade mucosal delayed-type hypersensitivity (DTH) reaction, and we explored ways to test this hypothesis, by measuring levels of soluble IL-2R and TNF α in jejunal fluids; and detecting the expression of mucosal markers of T cell activation, HLA-DR, DQ, and CD25. Non-coeliac patients with high total IEL counts also showed signs of mucosal inflammation, with HLA-DR expression in crypt epithelium, but no CD25+ cells in lamina propria.

Gluten sensitivity can only be tested by clinical trials of GFD and gluten challenge. A group of patients positive and negative for the CIA pattern were offered a trial of gluten-free diet, and a clinical response and evidence of histological changes (eg. decreased IEL counts) were found in some of them. One woman, clinically gluten sensitive and positive for three potential markers, voluntarily added extra gluten to her diet, and an enteropathy was found in a biopsy taken 5 months later.

Patients with one or more markers have potential coeliac disease, and if it is confirmed that they represent the only manifestation of gluten-sensitivity, then the current definition of coeliac disease should be changed.

AIMS OF THE STUDY

1. To characterize jejunal fluid aspirate as material for research in intestinal immunity.
2. To define intestinal immunoglobulins and antibodies in jejunal fluid from coeliac patients and controls. On the basis of this, a "CIA" pattern of intestinal antibodies was defined.
3. To assess the frequency of the CIA pattern amongst patients with similar symptoms to coeliacs, but normal histology, and their relative expression by other non-coeliac diagnostic groups.
4. To identify other putative markers of latent coeliac disease, their co-expression in patients and possible relationships with the CIA pattern.
5. To evaluate how the CIA pattern and other markers relate to the expression of clinical gluten-sensitivity, by trials of gluten-free diet and gluten loading.
6. To investigate possible immunopathological mechanisms responsible for the CIA pattern.

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Gastroenterology 1993, 104:1263-72.

Arranz E, Bode J, Kingstone K, Ferguson A.
Intestinal antibody pattern of coeliac

disease: association with τ/δ T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. **Gut** (in press).

LIST OF PRESENTATIONS BASED ON THIS THESIS

Arranz E, O'Mahony S, Ferguson A. Coeliac-like intestinal antibody pattern in non-coeliac patients with high normal jejunal gamma/delta expression- "Potential" coeliac disease?.
In: International Coeliac Symposium, Dublin, July 22-25, 1992.

Arranz E, O'Mahony S, Ferguson A. Expression of the intestinal antibody pattern of coeliac disease in patients with normal biopsy histology.
In: International Coeliac Symposium, Dublin, July 22-25, 1992.

Arranz E, Ferguson A. Jejunal fluid antibodies and mucosal gamma/delta IEL in latent and potential coeliac disease. In: 7th International Congress of Mucosal Immunology. Prague, August 16-20, 1992.

ABBREVIATIONS

α -1 AT	alpha-1 anti-trypsin
AEm	anti-endomysium antibodies
AGA	anti-gliadin antibodies
ARA	anti-reticulin antibodies
BB3	monoclonal antibody specific for the disulphide-linked form of T cell receptor
BLG	beta-lactoglobulin
CIA	coeliac-like intestinal antibody
CD3	pan-T cell marker (thymus-derived cells)
CD4	antigen expressed by helper/inducer T cells
CD8	antigen expressed by suppressor T cells
COE	coeliac
DAB	diaminobenzidine
DH	dermatitis herpetiformis
δ TCS1	monoclonal antibody specific for the non-disulphide-linked form of T cell receptor
dIgA	dimeric IgA
DTH	delayed type hypersensitivity
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
GALT	gut-associated lymphoid tissue
GFD	gluten-free diet

GLI	gliadin
H&E	haematoxylin & eosin
HML-1	monoclonal antibody specific for human intestinal lymphocytes
HLA	human major histocompatibility complex
HLA-DR	histocompatibility locus antigen (HLA)-DR
HLA-DQ	histocompatibility locus antigen (HLA)-DQ
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
HRP	horse radish peroxidase
hsp	heat shock protein
IEL	intra-epithelial lymphocyte
IgA	immunoglobulin A
IgM	immunoglobulin M
IFN γ	gamma-interferon gamma
IL-2R	interleukin-2 receptor
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
Ki-67	monoclonal antibody specific for a nuclear proliferation marker
LAK	lymphokine activated killer cell
LFA-3	lymphocyte function-associated antigen-3
LPS	lipopolysaccharide
mAb	monoclonal antibody
mIg	membrane immunoglobulin (B cells)
MHC	major histocompatibility complex

MMC	mucosal mast cell
NK	natural killer cell
NSAID	non-steroid anti-inflammatory drugs
OD	optical density
OTH	patients with other diagnoses (non-coeliac/DH)
OVA	ovalbumin
pIg	polymeric immunoglobulins (IgA, IgM)
PMSF	phenylmethyl sulphonyl fluoride
PVA	partial villus atrophy
PWM	pokeweed mitogen
SC	secretory component
sIgA	secretory immunoglobulin A
STVA	subtotal villous atrophy
RIA	radioimmunoassay
TAS+	TASIC software operating system
TBS	tris buffered saline
TCR	T cell receptor
TGF β	transforming growth factor-beta
Th1	T helper 1 subset
Th2	T helper 2 subset
TNF α	tumour necrosis factor-alpha
TVA	total villus atrophy
UCHL-1	monoclonal antibody specific for primed or memory cells
VCAM-1	vascular cell adhesion molecule-1

Note: the notation γ refers to 'gamma' (as in $\gamma\delta$ cells)

l	litres
ml	millilitres
ul	microlitres
kg	kilogrammes
mg	milligrammes
ug	microgrammes
pg	picogrammes
mm	millimetres
um	micrometres
U/ml	units per millilitre
IU/ml	international units per millimetre

Statistical notation

SD	standard deviation
p	probability
<	less than
>	more than
=	equal to
NS	not significant
*	$p < 0.05$ significant
**	$p < 0.01$ or $p < 0.01$ highly significant
r	regression coefficient

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SECTION ONE

REVIEW OF THE LITERATURE

CHAPTER I

A. THE HETEROGENEITY OF COELIAC DISEASE

Current definition of coeliac disease

Coeliac disease is currently defined as a permanent intolerance of the small bowel mucosa to gluten, in genetically predisposed individuals (Meuwisse 1970). Since there is great variability of malabsorption and heterogeneity of clinical presentation, the diagnosis is based on the presence of an abnormal jejunal biopsy while the patient is taking a normal diet. These changes affect the mucosal architecture, with crypt hyperplasia, and total or partial villus atrophy; but also a very characteristic increase of different populations of lymphoid cells in lamina propria, and a high density of IEL. Treatment with a GFD improves the histological appearances.

Epidemiology of coeliac disease

Coeliac disease is found preferentially in Europe, but it

may also affect individuals of European descent in other continents. The prevalence of the classic disease in Europe ranges between 1 in 5000 and 1 in 10000, though the actual figure may be higher (Auricchio 1988). Epidemiological studies have shown differences in disease frequency between countries or relatively close areas (eg Denmark and Sweden, or amongst groups of "European" genetic background in USA, where the prevalence is low), variation in the age of presentation, increased heterogeneity in clinical presentation (with milder and atypical forms), and discordance of monozygotic twins pairs. The data underline the importance of socio-economic or nutritional factors (eg feeding practices, dietary gluten content) as environmental triggers in genetically predisposed individuals (Cavell 1992, Greco 1992, Logan 1992, Loft 1993).

In most European countries, the trend in the last two decades has been towards a decrease in the incidence of coeliac disease, and a higher age at diagnosis, in late childhood or adolescence (Logan 1982, 1986; Maki 1988). However, in Spain, Portugal, and Poland, the cumulative incidence has increased, perhaps in relationship to an increased diagnostic awareness. Finland is a good example of the general trend, with over 60% of diagnoses made at school age or later, and a milder presentation in adults, even without abdominal symptoms (Maki 1992a). The

exception is Sweden, where the current incidence of 1/300 births, has been increasing in the last ten years (Ascher 1991).

Genetics of coeliac disease

Susceptibility to coeliac disease seems to be under multigenic control, and it has been associated with genes within the locus of the MHC that code for the human leukocyte antigens HLA-DR3, DR7, DQw2, and possibly others (Kagnoff 1990, 1992). These genes are located on the short arm of chromosome 6, which also contains genes for TNF α and β , and heat shock protein-70. In Europe, the majority of patients possess the extended HLA-DR3 haplotype that includes (A1) B8, DRw17, DQw2, DPw1 (Hall 1990a, Kagnoff 1992). The small minority negative for this haplotype are DR4, perhaps having a later onset of the disease, and more often atopic (Verkasalo 1983). The polymorphism of the HLA-class II proteins (α , β heterodimers) determines the spectrum of antigen binding affinities in each individual (Brandtzaeg 1991), and may modulate the immune responsiveness to gluten, either by thymic selection of the TCR repertoire or by HLA-restricted antigen presentation at local level.

Studies of molecular genetics have shown that within the HLA-D region, susceptibility to the disease is multigenic

as defined by analysis of "restriction fragment length polymorphism" in the DQ and DP subregions (Howell 1986, Niven 1987); although more than 95% of patients shared a combination of alleles encoding for the HLA-DQ $\alpha\beta$ heterodimer, identified as HLA-DQ A1*0501 and B1*0201 (Sollid 1989). The pair is located in cis position on the DR3 haplotype and in trans position in DR5/DR7 heterozygotes (Lundin 1990). Gluten-reactive HLA-DQ restricted T cells, have been isolated from the small bowel mucosa of coeliac patients (Lundin 1993). These alleles are more frequently expressed by healthy first degree coeliac relatives with high $\tau\delta$ IEL counts (Holm 1992). HLA-DR associated diseases (eg insulin-dependent diabetes, rheumatoid arthritis) also involve regulatory T cell abnormalities, increased humoral responses, and auto-antibodies.

Different forms of expression of coeliac disease

Within the framework of the current definition, clinical, pathological, epidemiological and immunological approaches reveal several forms of coeliac disease. Pathologically it is accepted that there is a degree of heterogeneity, and descriptive terms such as "flat mucosa", or "sub-total villus atrophy", are used by some pathologists for a cluster of features (villus and crypt sizes, epithelial cell damage, epithelial and lamina

propria cell infiltrates) which together characterize the enteropathy of coeliac disease. Quantitative histology (Ziegler & Ferguson 1984) and computerized image analysis (Marsh 1988) of biopsies taken during gluten withdrawal and challenge have shown that all of these features occur in a continuum with a flat, avillous lesion at one end of the spectrum and at the other a mucosa with normal villus and crypt architecture but an abnormally high density or IEL count (Marsh 1988). The latter would be reported as normal by many histo-pathologists without a specialist interest in gastro-intestinal pathology.

Intraepithelial lymphocytes and IEL counts

IEL form one of the largest T cell populations in the body, but their function is still unknown (Mowat 1990, Cerf-Bensussan & Guy-Grand 1991). This is a phenotypically heterogeneous population, though the majority (70-90%) are CD3+CD4-CD8+ and TCR $\alpha\beta$ + (Brandtzaeg 1989b, Jarry 1990). Many IEL lack other T cell-associated markers, eg CD5 and Thy-1, and they do not express conventional markers of activation, eg CD25 or HLA-class II antigens. About 10% of the total IEL population is CD4 positive. There are also double negative cells (CD4-CD8-) and a proportion of non-T lymphocytes (CD3-CD7+). The latter do not have markers associated with NK cells (CD16, CD56, CD57) and they do not have NK activity

(spontaneous cytolysis of K562 cells). The CD7 marker has also been associated with activated T cells (Selby 1983, Jenkins 1986, Malizia 1985).

Some IEL may migrate directly to gut epithelium at an early stage of ontogenesis where they remain thereafter. However, most IEL belong to a recirculating population which crosses the epithelial basement membrane from the lamina propria, and a great proportion have the HML-1 marker, perhaps related with the $\alpha E\beta 7$ integrin involved in IEL adhesion to enterocytes (Jarry 1990, Cepek 1993). Functionally, they have low proliferative activity in vitro with poor response to conventional T cell mitogens IL-2 and anti-CD3 antibody (Ebert 1990), and there is no strong evidence of cytotoxic activity in assays in vitro (Cerf-Bensussan & Guy-Grand 1991). IEL may be involved in the modulation of epithelial cell growth and marker expression (eg MHC-class II) by secreting specific cytokines (Mayer 1991). Almost 50% of IEL are CD45RO, an antigen-primed T cell population in close proximity to epithelial cells (Brandtzaeg 1989a, Haltensen 1990, Jarry 1990).

IEL counts in human jejunal biopsies were first reported by Ferguson & Murray (1971) in relation to the number of enterocytes, and were found to be increased in coeliac disease, cow's milk intolerance, giardiasis and tropical

sprue (Wright 1973, Ferguson 1977, Phillips 1979). High IEL counts have been found in crypt epithelium after gluten challenge in coeliac patients (Leigh 1985, Loft 1989, Marsh 1992) in parallel with an increased mitotic index, suggesting an increased cell turnover and activation (Ferguson & Ziegler 1986, 1987a). Intra-epithelial lymphocytes are also increased in experimental enteropathies without enterocyte loss in animals, gut allograft rejection, intestinal graft-versus-host reaction, local DTH responses to oral proteins, and giardiasis (Ferguson 1976, 1977; MacDonald & Ferguson 1976, Mowat & Ferguson 1981).

In untreated coeliac disease, activation markers are absent from IELs, and there is no change in the CD4/CD8 ratio, although CD8+ cells may have the CD5 marker or the T2 blast antigen (Selby 1983, Malizia 1985, Spencer 1989a, Jenkins 1986). The proportion of double negative CD4-CD8- cells increases in both treated and untreated coeliacs, in parallel with the population of $\gamma\delta$ IEL previously described (Spencer 1989a, Verkasalo 1990). Morphometric studies have shown that IEL infiltration is an early expression of gluten-sensitivity (Leigh 1985, Marsh 1988, 1989a, Loft 1989), and it is an early and common feature of the enteropathy induced by lamina propria T cell activation in organ culture of human fetal intestine, before histological damage (Monk 1988,

Brandtzaeg 1989a).

Experiments of gluten challenge confirmed the IEL infiltration (mainly CD3+ TCR $\alpha\beta$ + cells), which is paralleled by an enhanced expression of the adhesion molecules ELAM-1 and VCAM-1 (Sturgess 1990, 1992; Ensari 1993), and also led to HLA-DR expression, particularly by crypt epithelium (Ciclitira 1986a). In humans, there is indirect evidence that IEL can modulate HLA-DR expression by enterocytes (Scott 1987).

The development of the gluten-sensitive enteropathy

Some theories have suggested that patients with latent coeliac disease are merely at one end of a spectrum of the pathology of enteropathy with a minimal lesion of histologically normal appearance, and/or with a high IEL count (Marsh 1988, 1992). In studies of gluten challenge in treated coeliac patients, a pre-infiltrative (type 0) stage was added to describe DH cases whose unique abnormality is an abnormal pattern of intestinal antibodies (O'Mahony 1990a).

It is also possible that coeliac disease develops in a two-stage process, latent and fully expressed (O'Mahony 1990a). Aberrant immunity to gliadin is a relatively frequent occurrence (Auricchio 1988, Arnason 1992); it is

genetically restricted, and may be expressed in gut or skin. Mucosal immunological sensitization is an invariable feature of coeliac disease, but is not the precipitating factor for the expression of the full intestinal lesion, as it was also shown in an animal model of gluten-sensitive enteropathy (Troncone & Ferguson 1991a). A second factor, such as an episode of hyperpermeability, nutrient deficiency, increased dietary intake, impaired intraluminal digestion of ingested gluten, adjuvant effects of intestinal infection, and a non HLA-associated gene may drive the enteropathy from minimal/latent to overt, either by immunological mechanisms or by direct ancillary effects on enterocytes (Ferguson 1993).

The clinical effects of a GFD or gluten challenge may parallel the effect observed in biopsy, but mucosal changes may be even more sensitive. Symptomatic patients with a morphologically normal mucosa and high IEL counts have been shown to respond to a GFD (Cooper 1980). There is also a report of first-degree relatives of coeliac patients with normal biopsy where an increased gluten intake did not lead to mucosal damage (Polanco 1987). Patients selected by high serum AGA levels but a normal biopsy did show a high IEL count (O'Farrelly 1987). A similar group identified by these criteria had abdominal symptoms and iron deficiency anaemia more often than a

control group (Arnason 1992).

Latent coeliac disease

Latent coeliac disease refers to those patients who have normal jejunal biopsy histology while taking a normal diet, but at some other time, before or since, have been shown to have typical, severe gluten-sensitive enteropathy which recovers on a GFD (Ferguson 1992). The concept was first described in two patients with dermatitis herpetiformis (DH) who developed a typical gluten-sensitive enteropathy weeks after starting a trial of 20 g of gluten added to their already gluten-containing diet (Weinstein 1974). Nearly all studies have been performed in DH patients and first-degree relatives of coeliacs, and these have confirmed the existence of this condition (Doherty & Barry 1981, Ferguson 1987b, Chorzelski 1988).

Dermatitis herpetiformis as true latent coeliac disease

DH and coeliac disease are both gluten-sensitive disorders that share a similar HLA haplotype (Sollid 1989, Hall 1990b). A high percentage of DH patients have an enteropathy identical to coeliac disease, in some of them this is only expressed by a high IEL count (Marsh 1989b), and a small proportion have a completely normal

jejunal morphology and function (Gawkrödger 1991). The intestinal abnormalities of DH (ie. high IEL counts), and the skin lesions, even in the cases with normal mucosa, respond to a GFD (Reunala 1977). Experiments of gluten loading in patients with normal mucosa led to the development of enteropathy (Ferguson 1987b). Therefore by the definition, DH patients with normal jejunal biopsies are true latent coeliacs. The latter group may also include first degree relatives of coeliacs (Marsh 1990), discordant monozygotic twins for the disease (Salazar 1987, Kamath & Dorney 1983), and patients with recurrent oral ulcers (Wray 1981) or gluten-sensitive diarrhoea (Cooper 1980).

Cases of latent coeliac disease in the literature

McConnell & Whitwell (1975). A 40-year-old woman with megaloblastic anaemia due to folic acid deficiency underwent a jejunal biopsy which was normal. She was diagnosed as having a nutritional deficiency. Five years later, she presented with diarrhoea at another hospital, where a second jejunal biopsy (without knowledge of the first one) showed a flat mucosa. She had a good response to a GFD.

Egan-Mitchel (1981). An 8-year-old girl was investigated because of small stature and iron deficiency, her

terminal duodenal biopsy was normal (IEL:26%) while eating 10 g of gluten daily. She was re-investigated 14 months later because of poor growth, persistence of tiredness, and lack of response to iron supplements. The second jejunal biopsy showed villous atrophy (IEL:78%), which resolved on a GFD (IEL:35%), and relapsed 4 months later after gluten challenge (IEL:51%).

Rolles (1981). A 4-year-old girl was investigated because she had loose stools, mild abdominal distension, slightly abnormal growth ratio, and atopic eczema but her jejunal biopsy was normal. Her mother had coeliac disease. Twenty two months later, she was referred with similar symptoms and challenged with 10 g.b.d. of gluten for 2 months before being reviewed. The second jejunal biopsy was flat, and a quick improvement was observed after treatment with a GFD.

Kamath (1983). One of the monozygotic twins had a diagnosis of coeliac disease when he was 11 months old. At that time, the second twin was asymptomatic and had a normal biopsy. However, at 9 years of age, a jejunal biopsy performed because of inadequate weight gain for 12 months, showed an atrophic mucosa.

Salazar de Sousa (1987). A discordant monozygotic twin of a coeliac patient. The first jejunal biopsy was taken at

3 years/10 months of age because of his brother's previous diagnosis, but it was normal and he had no symptoms. Seven years later, he presented with weight loss, abdominal distension, anorexia, and fatigue, and a second jejunal biopsy showed a subtotal villous atrophy. The diagnosis was confirmed with trials of a GFD and gluten challenge.

Marsh (1989a). The first case was a 59-year-old woman with 20 years history of malabsorption and macrocytic anemia, and two previous morphologically normal jejunal biopsies, in which morphometric analysis showed an IEL infiltration. Five months later, she presented with an episode of watery diarrhoea and weight loss, and a third jejunal biopsy showed a villous atrophy that improved on a GFD. The second case was a 40 year old woman with a first (normal) jejunal biopsy performed because of megaloblastic anemia due to folate deficiency. Five years later, the biopsy was flat, followed by a good response to a GFD.

Maki (4 cases in 1990, 1 case in 1991).

A 0.9-year-old girl was studied because of poor weight gain, but she had no GI symptoms and no family history of coeliac disease. Her first jejunal biopsy was normal (IEL:34%) and had slightly high serum IgG AGA. After 3.5 years, a second biopsy performed because of high levels

of IgA and IgG AGA, and IgA of both ARA/AEm, showed STVA which resolved on a GFD.

The second case was a 2.3-year-old boy, whose brother was coeliac. He had intermittent loose stools. The first jejunal biopsy was normal (IEL:31%) and serum antibodies were negative. At 7.6 years of age, he had no abdominal symptoms, but a second biopsy performed because of high ARA-IgA levels showed STVA.

The third case was a 11.4-year-old boy without GI symptoms, suspected of having DH, and a sister with coeliac disease. His jejunal biopsy was normal (IEL:21%), and ARA antibodies and skin biopsy for DH were negatives. As part of a family study, when he was 17.5 years old, a second biopsy showed STVA, and levels of IgA and IgG ARA/EMA were positive. A GFD normalized these parameters after 6 months.

A 32-year-old woman had diarrhoea and was suspected of having ulcerative colitis. She was involved in a family study because of a coeliac child, and at that time her jejunal biopsy was normal. Nine years later she was asymptomatic, and in a similar study her biopsy showed PVA (IEL:41%), which was normalized after a GFD.

The last case refers to an asymptomatic 35-year-old man with a coeliac son and a first normal jejunal biopsy

(IEL:18%). Two years later, a second biopsy taken because of high serum IgA AGA and worsening of a preexisting epilepsy showed villous atrophy and crypt hyperplasia (IEL:56%). The lesion resolved after 6 months of GFD (IEL:32%). Counts of epithelial CD3+ cells (81/mm), TCR $\alpha\beta$ + (49/mm) and TCR $\gamma\delta$ + cells (31/mm) were performed in frozen sections from the stored first biopsy.

Catassi (1991). A 22-month-old girl diagnosed as having insulin-dependent diabetes mellitus (IDDM). She had no GI symptoms or family history of coeliac disease (only HLA-B8,DR3), and a jejunal biopsy performed because of high serum IgG AGA showed a normal architecture. She had persistent high serum AGA levels and occasional abdominal problems. Two years later, a second biopsy showed a total villous atrophy, but also serum AGA antibodies were positive and the sugar permeability test was abnormal. GFD and gluten challenge confirmed the diagnosis.

Collin (1993). Seven cases (5 males, 2 females; age range: 22-66) had a jejunal biopsy taken 1-5 years after a first normal jejunal biopsy (while on a normal diet) because of abdominal symptoms in a follow-up study. The biopsies showed signs compatible with coeliac disease. The IEL count was high in only one of the first biopsies, but all were positive for serum IgA-ARA or IgA AGA titers.

TABLE I.1. CASES OF LATENT COELIAC DISEASE IN THE LITERATURE

(first of two pages)

	SEX	AGE	<u>FIRST NORMAL BIOPSY</u>		IEL COUNT	IEL % ⁺	<u>SECOND BIOPSY</u>		HISTOLOGY
			REASON				REASON	AGE	
McConnell	f	40	Anaemia	ND	ND		Diarrhoea	45	"Flat"
Egan- Mitchell	f	8/9	Small. Anaemia	26%	ND		Poor growth	9/11	TVA
Rolles	f	4	Coeliac mother	ND	ND		Abdominal distension	5/10	TVA
Kamath	m	-/11	Monozyg. twin	ND	ND		Poor growth	9	TVA
Salazar	m	3/10	Monozyg. twin	ND	ND		Weight loss Anorexia	10	TVA
Marsh a	f	62	Malabs. "high" Anaemia	ND	ND		Diarrhoea	64	TVA

TABLE I.1. (continued)

<u>FIRST</u> <u>NORMAL</u> <u>BIOPSY</u>						<u>SECOND</u> <u>BIOPSY</u>			
	SEX	AGE	REASON	IEL	IEL	REASON	AGE	HISTOLOGY	
				COUNT	7δ+				
Marsh	b	f	40	Megalob."high"	ND	Anaemia	45	TVA	
			Anaemia			Fol.def			
Maki	a	f	0.9	Poor	34%	ND	High AGA,	3.5	TVA
			wt gain				ARA/AEm		
	b	m	2.3	Coeliac	31%	ND	High	7.6	TVA
			brother				IgA ARA		
	c	m	11.4	Coeliac	21%	ND	Familiar	17.5	TVA
			sister				study		
	d	f	32	Diarrhoea	ND	ND	Familiar	41	PVA
			Coe.child				study		
Maki		m	35	Coeliac	18%	31/mm	High	37	TVA
			son				IgA AGA		
Catassi		f	1/10	IDDM	ND	ND	High AGA	3/10	TVA
			High AGA				Abn Permb		
Collin	(5/2f)	22-66	High ARA/AEm		ND		Follow up	1-5ys	P/TVA

B. IMMUNOGLOBULINS AND ANTIBODIES IN JEJUNAL SECRETIONS

Immunoglobulins A and M, and the secretory component

Secretions are characterized by a great proportion of locally synthesized polymeric immunoglobulins. Jejunal fluid contains the largest amount of polymeric immunoglobulins (IgA and IgM), present in the upper small bowel. Their origin is by local synthesis in lamina propria and they reach the lumen by an active transepithelial mechanism mediated by secretory component, involving 98% of pIgA, and 99% of pIgM (Brandtzaeg 1981, Mestecky & McGhee 1987), whereas most plasma proteins may reach the lumen by passive leakage from lamina propria capillaries (Jonard 1984). Polymeric IgA represents 92% of jejunal IgA, almost entirely bound to secretory component (Delacroix 1982). The higher number of IgA2-containing cells in jejunal lamina propria, determine that 35% of jejunal fluid IgA is of IgA2 subclass (Kett 1986). The ratio between IgA1 and IgA2 subtypes may vary according to the area of intestine and type of antigen.

Secretory IgA (sIgA) is a dimer that forms a complex with

part of the transepithelial receptor (SC), and a disulphide-linked polypeptide or J chain, and represents the main immunoglobulin in secretions (Mestecky 1987). IgA-producing cells in the intestinal mucosa represent between 80-90% of all plasma cells (Brandtzaeg 1991), and there are also specific regulatory mechanisms in mucosal lymphoid organs that lead to the generation of memory B cell clones with preferential secretion of IgA. B lymphocytes express membrane immunoglobulins as antigen receptors (normally IgM), they proliferate after stimulation and some of them (memory cells) will differentiate to immunoglobulin-secreting cells after migration to mesenteric nodes and other mucosal areas, where they undergo terminal differentiation (Strober 1990).

IgM is the major secretory immunoglobulin not only in the intestine of children (Savilahti 1972), individuals with IgA deficiency (Eidelman & Davis 1968), and during the early local immune response (Girard & Kalbermatten 1970), but also in young animals (Porter 1977). Pentameric IgM (pIgM), like dimeric IgA, binds to polymeric Ig receptor (SC) (Weicker & Underdown 1975, Brandtzaeg 1977, Richman & Brown 1977); and both dIgA and pIgM are secreted into the lumen by a mechanism of epithelial transcytosis mediated by SC (Brandtzaeg 1985, Conley & Delacroix 1987). This mechanism may be upregulated by interactions

between epithelium and activated T cells (Scott 1981, Sollid 1987, Kvale 1988); but it seems not to be affected by crypt hyperplasia. Polymeric IgM may display stronger non-covalent interactions with SC, which has a higher affinity than dimeric IgA (Brandtzaeg 1985, Bouvet 1990), and therefore locally-produced IgM might block, to some extent, the IgA transepithelial transport by competing for the receptor.

Functions of secretory immunoglobulins

Secretory IgA functions as a first line of defence, preventing the adherence and absorption of microorganisms to the epithelium, by immune exclusion (Svanborg-Eden & Svennerholm 1978, Taylor & Dimmock 1985). Recently, it has been suggested that IgA may be active not only in secretions, but also within the epithelium, by intracellular neutralization of virus; and in the lamina propria, by transporting immune-complexes to the lumen and therefore preventing antigens and toxic products from reaching the circulation (Mazanec 1993). Because of this (probable) excretory function, and its known poor activity in triggering complement (Pfaffenbach 1982), IgA has non-inflammatory activity, protecting the mucosa against potentially damaging mechanisms involving complement activation (Conley & Delacroix 1987, Mestecky & McGhee 1987, Brandtzaeg 1991).

Unlike IgA, IgM may opsonize antigens through complement activation, but this effect may be down-regulated by the abundance of IgA and its effective competition for antigen. Subepithelial deposits of C3b and terminal complement complex have been found in coeliac disease, and these are particularly increased after gluten challenge (Haltensen 1992a). The finding of these deposits was correlated with the number of mucosal IgG+ plasma cells, and with serum IgG antigliadin antibodies. It may be also related to the increased local synthesis of IgM found in coeliacs, that would form immune-complexes after competing for the antigen with IgA.

Measurement of secretory IgA in jejunal fluids

The measurement of secretory IgA, as % of the total IgA bound to SC in jejunal fluids, may serve as an indirect indication of local immunoglobulin secretion. Secretory component is a trans-membrane glycoprotein receptor for polymeric immunoglobulins, expressed by intestinal epithelial cells. It has been studied by immunohistochemistry in frozen sections (Brandtzaeg 1974, Brown 1976, 1977); and by ELISA, RIA, and other methods in secretions, either as a free molecule or bound to J chain positive polymeric immunoglobulins (IgA and IgM) (Delacroix & Vaerman 1981).

A non-competitive ELISA method has been used to measure sIgA, sIgM, and SC, in serum, but also in culture supernatants and lysates (Kvale & Brandtzaeg 1986, 1988; Sollid 1987, Wood 1987). To specifically measure sIgA, sIgM can be removed by absorption using small immunosorbent columns containing specific anti-IgM antibodies (Bartholomeusz 1989). An alternative method coats microtiter plates with specific anti- α chain and uses an anti-SC conjugate as a secondary antibody (Ishiguro 1981, Hjelt 1988). Low levels of free-SC were found in most biological fluids (sera, urine, whole saliva, jejunal fluid) using a sandwich ELISA method with different specific mAbs for sIgA and sIgM to measure free and bound SC (Vincent & Revillard 1988). The potential advantage of using monoclonal (instead of anti- α chain) antibody, is the lack of interference between SC and sIgA measurements, and also that IgM is not detected. However, the specificity of monoclonal antibodies and the results obtained have been criticized (Kvale 1988).

Secretory IgA and IgM specific antibodies to gliadin and other food proteins have also been tested in serum (Volta 1985) and jejunal fluid samples (Volta 1990) by coating microtiter plates with crude antigen and using an alkaline phosphatase-conjugate anti-SC as secondary antibody. A similar test has been used by others to

measure antigliadin and specific sIgA antibodies in serum from coeliac children, but the polyclonal SC peroxidase-conjugate antiserum was different (Arranz 1986, Blanco 1989).

Regulation of immunoglobulin production in intestine.

Antigen exposure in gut-associated lymphoid tissue -GALT- induces the generation of secondary or memory B cells specifically committed to the IgA isotype (Cebra 1984). Most oral antigens are delivered to the Peyer's patches, where germinal centres contain immature IgM+ B cells (60%), macrophages and other antigen-presenting cells. This microenvironment provides the necessary stimulation for B cell development and the activation of a particular T helper subset (Weinstein & Cebra 1991, Biewenga 1993), that, either by direct contact, or by lymphokine release, together with Peyer's patch stromal and dendritic cells, will control the first stages of B cell proliferation and isotype switching to functional IgA+ B cells (Kawanishi 1983a,b; Spalding & Griffin 1986).

During primary and secondary responses, distinct subsets of helper (CD4+) T cells may regulate the antibody response. Antigen is not directly involved in the generation of antibody specificity in the course of B-

cell development (rearrangement and deletion of DNA gene segments encoding the heavy and light immunoglobulin chains), however, terminal differentiation requires B cell activation by antigen, that will determine the selection and expansion of particular cell clones in mucosa (Strober 1990). Interactions between antigen, specific B cells and different subsets of lymphokine-secreting T cells may determine the isotype and immunoglobulin subclass produced. In mice, primary stimulation of spleen cells leads to IL-2 and IFN γ production, whereas priming and subsequent in-vitro restimulation induces IL-4 and IL-5, the latter specifically in mucosa (Swain 1988, 1991).

Development of mucosal plasma cells

I. Switch differentiation.

There is a unique population of T cells located in the Peyer's patch germinal centres which direct the switch of IgM $^{+}$ to IgA $^{+}$ specific isotype B cells. These T cells act either by direct contact with IgM $^{+}$ B cells or by cytokine secretion, and induce DNA-binding proteins to make accessible the 'switch sites' allowing transcription and rearrangement of the (C α) gene for the constant region of the α chain (McGhee 1989, Strober 1990). In the early stages of this process T cells and some lymphokines may

be involved. Transforming growth factor β induces IgA secretion in-vitro, and its effect is enhanced by the addition of IL-2 or IL-5 to LPS-stimulated B cells in culture (Sonoda 1989, Coffman 1989). However, TGF β may not be a primary switch factor, but secondary to other stimuli (acting on already committed IgA-cells). IL-4 may also facilitate the switch from IgM+ B cell to IgA+ B cells.

II. Postswitch differentiation.

Terminal differentiation and clonal expansion of committed IgA+ B cells probably takes place in mesenteric nodes and lamina propria. After antigen stimulation in Peyer's patches, lymphoblasts form a recirculating reservoir of rapidly switching IgA+ B cells, whose migration is controlled by interactions of lymphocyte adhesion molecules with site-specific addressins on high endothelial venules (Kraal 1983, Picker & Butcher 1992). These lymphoblastic cells undergo differentiation into antibody-secreting plasma cells in response to local factors, eg IL-5, IL-6 and IFN γ (Zeitz 1988), which may be delivered by specific T cells bearing IgA-Fc receptors. Different lymphokine-secreting T cell subsets may be prevalent at mucosal sites, but cytokines may also be isotype-specific in B cell differentiation. IL-5 interacts directly with specific receptors on IgA-

committed B cells, enhancing IgA (and IgM to a lesser extent) antibody responses to LPS (Coffman 1987, Harrison 1988). Optimal IgA responses may be provided by their cooperation with other signals, eg IL-6.

Both human and mouse IL-5 and IL-6 increase the in-vitro IgA secretion from isolated murine mucosal cells (Beagley 1989). In coeliac disease, the local secretion of IL-5 may explain in part, the increased synthesis of secretory IgA antibodies. Coeliac patients also have an increased eosinophil population in the intestinal mucosa (Marsh & Hinde 1985), and IL-5 is involved in eosinophil differentiation, though these cells may also be a source of IL-5 (Desreumaux 1992). It is known that IgA complexes may trigger human eosinophil degranulation through IgA receptors, leading to local release of cytotoxic protein and tissue damage (Colombel 1992).

Circulating antibodies to foods in coeliac disease

IgG antibodies have been found to many food antigens, whereas IgA antibodies have been little studied, except for gliadin. A great proportion (80%) of these antibodies in adults and children are of the IgA1 subclass, probably of bone-marrow origin (Mascart-Lemone 1988, Arranz 1986, Engstrom 1992). In untreated patients, both subclasses are enhanced, specially IgA1 with

increased IgA1/IgA2 ratio (Elewaut 1989). Sera from coeliacs and normal individuals contain antibodies with specificity for a variety of gliadin subunits, and jejunal fluids seem to have an even broader and different range (Skerritt 1987). However, there is also a report of a coeliac patient with hypogammaglobulinemia, absence of mucosal plasma cells and no serum antigliadin antibodies (Webster 1981).

Measurement of anti-gliadin antibodies in serum has been proposed as a non-invasive and reliable test for screening of patients suitable for biopsy, and a useful tool for monitoring dietary compliance, especially in treated children (Volta 1985, Savilahti 1983, Troncone & Ferguson 1991, McMillan 1991, Maki 1992b); however 20-30% of patients with active disease may be negative for this test (Volta 1990, Scott 1992). IgG antibody levels are more sensitive, but the specificity of IgA is higher and correlates better with the presence of enteropathy. Better results can therefore be obtained with a combination of both antibody titers (Savilahti 1983, Arranz 1986). Treatment with gluten restriction decreases IgA antibodies to within a normal range in a short period of time, whereas IgG antibodies remain high longer, even after resolution of the mucosal lesion (Savilahti 1983, Kilander 1987).

It has been suggested that anti-gliadin antibody production may be under genetic control (Mearin 1984, Weiss 1983), and therefore the aberrant humoral response to gliadin may be explained by an immunoregulatory defect, eg suppression (Pignata 1985, Corazza 1986). This hypersensitiveness seems to be independent of the HLA genotype associated with coeliac disease (DR3, DQ2 haplotype), though HLA-DR2 expression was found mainly in individuals with high antibody titers. These antibodies may be associated with gluten sensitivity in the absence of coeliac disease (Corazza 1992), and they have been found to be increased (sometimes with abnormal intestinal permeability and/or high IEL counts) in MHC-related diseases mediated by immunological mechanisms, such as DH, IgA nephropathy, rheumatoid arthritis, sarcoidosis and recurrent oral ulceration. In the latter, high anti-gliadin antibodies may identify patients who will respond to GFD (O'Farrelly 1991a,b).

Antibodies in jejunal fluids

Early reports found precipitins to gliadin and other dietary antigens in duodenal fluid of untreated coeliacs (Katz 1968, Herscovic 1968, Ferguson & Carswell 1972). It is generally agreed that the intestinal fluid in untreated coeliac disease patients contains high levels both of IgA and IgM anti-gliadin antibody, and that in

contrast to serum antibodies, levels of intestinal antibodies remain high after healing of enteropathy in treated coeliacs (LaBrooy 1986, Volta 1988, Colombel 1990, O'Mahony 1991a, Lavo 1992). Intestinal antibody levels do not correlate with those of serum (O'Mahony 1991a, Kelly 1991), and this independence is also shown by a different isotype pattern of antibody response to gliadin. Monomeric IgA (mainly IgA1) and IgG predominate in serum, while polymeric IgA (higher proportion of IgA2) and IgM, bound to SC predominate in intestinal fluid (Jonard 1984, Scott 1992).

High counts of lamina propria plasma cells have been found in coeliac mucosa of both untreated adults and children, particularly IgM-containing cells whose density was similar to IgA+ cells (Douglas 1970, Lancaster-Smith 1974, Baklien 1977, Scott 1984, Dhesi 1984, see results in Chapter XI). The increased immunoglobulin production is not limited to food protein antibodies. Treated patients have fewer plasma cell numbers than untreated coeliacs, but a greater number than controls (Baklien 1977). In children, however, similar results were found in treated patients and controls, suggesting that they may have a higher potential for mucosal normalization (Scott 1980a, Savilahti 1972). In a recent investigation of intestinal immune responses to an enteric vaccine, coeliacs generated high levels of intestinal IgM

antibodies to a bacterial antigen, Cholera toxin B subunit (O'Mahony 1990b).

These in-vivo results have been confirmed by in-vitro studies where local secretion of antibodies has been found in organ culture models of jejunal biopsies (Wood 1987, Fluge & Asknes 1983, Ciclitira 1986b) or studies with isolated intestinal lymphocytes (Crabtree 1989a). Using the ELISPOT technique for the enumeration of class specific antibody-secreting cells, untreated coeliacs had a higher number of anti-gliadin spot-forming cells compared with controls (Lycke 1989), though the anti-gliadin specificity represents only a small proportion of Ig-producing cells. It has been reported that almost 50% of the net increase of IgA and IgM in an in-vitro model of gluten challenge, is due to synthesis of antigliadin antibodies (Falchuk & Strober 1974). However, others have found that specific antibodies account respectively for the 2.1, 12.1, and 4.1% of the total IgA, IgM and IgG production by jejunal biopsies (Ciclitira 1986b).

Circulating anti-reticulin and anti-endomysium antibodies

Coeliac and DH patients have serum antibodies against the connective tissue surrounding the intestinal smooth muscle. These antibodies are detected by indirect immunofluorescence on cryostat sections from monkey oesophagus

(primate-type or anti-endomysium antibodies), or rat tissues (rodent-type or anti-reticulin antibodies) (Chorzelski 1983, Burgin-Wolff 1991; Maki 1991b, 1992a). Anti-endomysium antibodies of IgA class have a high specificity in both diseases, where they have been detected in 90-100% of untreated adults and children with coeliac disease (Chorzelski 1983, Kumar 1989, Garrote 1992), and in 70% of DH patients. The specificity is highest in patients with mucosal atrophy (Acetta 1986). The antibodies have not been found in other skin or gut diseases (Kumar 1989), and it is known that anti-gliadin antibodies also bind to endomysial antigenic sites (Beutner 1986). The detection of ARA/AEm antibodies may be an useful screening test to identify mild or atypical forms of the disease, eg amongst relatives of coeliac patients (Hallstrom 1989, Watson 1992). High antibody titers have been found in some relatives of coeliac patients at the time of the first normal biopsy, before the development of a gluten-sensitive enteropathy (Maki 1990).

Anti-reticulin and anti-endomysium antibodies may have a similar origin, perhaps as human auto-antibodies triggered by gluten ingestion in genetically predisposed individuals. They have different cross-reactivity when tested in animal tissues, ARA in mice, and AEm in monkey tissues (Maki 1991b, 1992a). Coeliac sera react with

human liver, jejunum, lung, spleen, thymus and pancreas; however, ARA specificities can be removed separately by absorption with rat liver homogenates (Hallstrom 1989). These antibodies of IgA class have also been found in jejunal juice (Mawhinney & Lowe 1975). Other disease-specific circulating antibodies have been identified in children (Karpati 1990), these bind to normal human jejunum and have similarities with ARA and AEm but not with anti-gliadin antibodies. The positivity for IgA-class ARA in patients with normal biopsy histology has a high predictive value for the development of enteropathy, and ARA and AEm antibodies may therefore be used as a marker of genetic predisposition in both silent and latent coeliac disease, especially amongst first-degree relatives of coeliacs (Maki 1992b, Collin 1990, 1993; Holm 1993a).

C. EXPRESSION OF THE $\tau\delta$ T CELL RECEPTOR

The T cell antigen-receptor

Immune cells may recognize and interact with a great variety of foreign antigens by two polymorphic structures: immunoglobulins and the T cell receptor. The TCR is a complex molecular structure consisting of a clonally variable and MHC restricted antigen binding subunit, the disulphide-linked $\alpha\beta$ heterodimer, and the five-chain complex of the CD3 molecule, to which the $\alpha\beta$ heterodimer is non-covalently bound (Brenner 1985, Marrack & Kappler 1986). The generation of the functional repertoire for antigen recognition is the result of positive and negative selection during intra-thymic maturation (Fawlker & Pardoll 1989). T cells $\alpha\beta$ + recognize antigen "processed" by macrophages and other antigen-presenting cells and bound to MHC molecules on their surfaces.

A second isotype of TCR was identified after the finding of a third gene (located on the short arm of chromosome 7) and its corresponding τ glycoprotein which was found to be expressed with the δ chain (Saito 1984, Brenner 1986).

The TCR α , β , γ , and δ loci include V (variable), J (joining) and D (diversity) gene segments similar to those described for the B lymphocyte receptor, which undergo somatic rearrangements to generate functional genes (Tonegawa 1983, Allison & Havran 1991). Both TCR $\alpha\beta$ and $\gamma\delta$ heterodimers are expressed in a mutually exclusive manner, but have high structural homology and are associated with the same signal transduction complex (CD3) (Raulet 1989). They represent two functionally distinct T cell lineages with a different recognition structure. The specificity, MHC restriction and function of TCR $\gamma\delta$ cells are still largely unknown (Tonegawa 1984, Raulet 1989b, Haas 1993).

Differences between TCR $\alpha\beta$ and $\gamma\delta$ heterodimers

Most of the human T cells express the $\alpha\beta$ form of TCR, whereas $\gamma\delta$ T cells are less common, representing between 1 and 20% of the total T cell population in peripheral blood, less than 5% in lymphoid organs, and up to 10% of T cells in the intestinal epithelium and lamina propria (Bottino 1988, Faure 1988, Groh 1989, Bucy 1989, Trejdosiewicz 1989, Spencer 1989a). Flow cytometric analysis of normal human intestine has revealed that most $\gamma\delta$ IELs are CD8+ and almost 20% of these have the CD8 $\alpha\alpha$ homodimer (Lynch 1993). Studies in animals showed that, unlike $\alpha\beta$ T cells, a high number of $\gamma\delta$ cells do not

express CD4 or CD8 molecules, and only a few are CD4 or CD8 positive (Lanier 1986, Groh 1989, Bucy 1989, Morita 1991, Moretta 1991). In mice, a great proportion of the IEL $\tau\delta$ + express CD8 $\alpha\alpha$ homodimers (Guy-Grand 1991). The lack of CD4 or CD8 molecules suggest that $\tau\delta$ + cells may recognize antigen in its native form or presented by molecules distinct to class I or II, eg class I-like monomorphic CD1 molecules (Porcelli 1989, Faure 1990). However, conventional MHC restriction elements, such as HLA-A2, DR3/7 or HLA-DQA1/DQB1 heterodimers, have also been reported (Strominger 1989, Ciccone 1989, Spits 1990, Bosnes 1990).

Phenotypically, $\tau\delta$ T cells can be divided into two subsets, which may follow distinct developmental pathways (Groh 1989, Falini 1989, Haas 1993). They differ in the stage of appearance during ontogeny and in the set of τ and δ genes used. The V δ 1/J δ 1 subset is recognized by the mAb δ TCS1 specific for the non-disulphide-linked form of heterodimer. This subset predominates in the thymus throughout life (foetal thymus express $\tau\delta$ T cells early in ontogeny), and represents approximately 50% of the peripheral blood lymphocytes in children <1 year, 20% in adults (Ho & Campana 1991), and 50 to 70% of TCR $\tau\delta$ + cells in intestine. The V δ 2 subset, recognized by the mAb BB3, becomes predominant in the periphery within the first month of life, and represents 70 to 80% of the

peripheral blood lymphocytes. These cells use the disulphide-linked form of receptor and are absent in the thymus (Bottino 1988, Spencer 1989a, Brandtzaeg 1989a, Haas 1990).

Morphologically, TCR $\gamma\delta$ + cells are similar to large granular lymphocytes, showing an extended cytoplasm and numerous electron-dense granules (primary lysosomes), and these features have been found in peripheral blood derived TCR $\gamma\delta$ + clones (Moretta 1991, Arancia 1991). TCR $\gamma\delta$ + cells also have similarities with NK cells as described in studies with isolated murine IELs, and they may undergo similar changes to those observed in LAK cells after IL-2 activation (Lefrancois & Goodman 1988, Viney 1990).

Function and antigen recognition repertoire of TCR $\gamma\delta$ + cells

TCR $\gamma\delta$ + cells might represent either the non-essential evolutionary remains of a primitive T-cell population that later evolved into $\alpha\beta$ + T cells, or a supplementary subset involved in some aspects of immune defence not covered by $\alpha\beta$ T cells (O'Brien 1991). TCR $\gamma\delta$ + cells are frequently localized into various epithelia (Goodman & Lefrancois 1988, Bonneville 1988, Bucy 1989, Groh 1989, Itohara 1990) and this characteristic localization

together with its restricted V gene usage suggests a separate and specific role in immunological surveillance that might be mediated by the recognition of stress-induced self-antigens, rather than foreign antigens (Janeway 1988, Allison & Havran 1991).

The fact that human TCR $\gamma\delta$ + cells may recognize a different group of ligands, sometimes localized in areas susceptible to trauma and pathogen entry, such as the villus epithelium, and that these subsets have a restricted V gene usage for each tissue localization, suggest an heterogeneous function. This suggested function of TCR $\gamma\delta$ + cells is based on their distribution in vivo and their functional capabilities in vitro (Haas 1990, 1993; Raulet 1989a, Born 1990, Janeway 1988).

Protective role. It has been suggested that TCR $\gamma\delta$ + cells might play a special role as a first line of defence at mucosal sites by the recognition of infected, transformed or damaged epithelial cells. Recognition of mycobacteria, autologous shock proteins, and superantigens such as staphylococcal enterotoxin A, has been observed (Raulet 1989b, Rust 1990, Born 1991, Porcelli 1991). These cells also accumulate in human chronic infectious lesions, such as rheumatoid synovitis (Modlin 1989, Brennan 1989, Sioud 1991). Early in life, $\gamma\delta$ + cells may provide a non-HLA-restricted local and

rapid response to common antigen determinants on pathogens or newly damaged self tissues. Later, when more elaborate protective responses are developed (by $\alpha\beta$ cells), TCR $\gamma\delta$ cells may help to initiate and localize specific immune responses, or stimulate the formation of granulomas (Allison & Havran 1991, Born 1991, Haas 1993).

Effector function. TCR $\gamma\delta$ cells may be related to non MHC-restricted cytotoxicity specific for alloantigens and heat shock proteins (Patel 1989, Brenner 1988, O'Brien 1989), and against tumour cells (Ciccione 1988, Fisch 1990). However, it is not known if they mediate tissue damage in vivo. TCR $\gamma\delta$ cells may produce high levels of lymphokines, but this is not related to CD4 or CD8 expression, or to the cytotoxic activity (Patel 1989, Christmas & Meager 1990, Morita 1991). There is an expansion of TCR $\gamma\delta$ cells in the intestine of patients with coeliac disease, but this does not affect any particular T cell clone, nor is it known if they play any pathologic role in the disease (Spencer 1989a, Tredjosiewicz 1991, Rust 1992, DeLibero 1993).

Regulation of B cell maturation. TCR $\gamma\delta$ cells may be involved in B cell development and provide help for immunoglobulin synthesis by production of a particular pattern of cytokines (Morita 1991). Stimulated murine gut epithelial TCR $\gamma\delta$ clones can secrete IL-2, IL-5, IFN γ and

TNF α , though perhaps in lower amounts than $\alpha\beta$ CD4 cells (Christmas & Meager 1990, Fujihashi 1989, 1992; Tagushi 1991, Barrett 1992).

Intra-epithelial TCR $\gamma\delta$ + cells: differences between mice and humans

The epithelial predominance of the IEL $\gamma\delta$ + subset in mice, and the preferential usage of different V γ and V δ gene segments determined by the local epithelial micro-environment, eg V γ 6 in gut, V δ 1 in the reproductive tract (Asarnow 1988, Jarry 1990, Hohara 1990), suggests that $\gamma\delta$ + cells may have a role in maintenance of the epithelial integrity (Goodman & Lefrancois 1988, Bonneville 1988, Janeway 1988, Allison & Havran 1991). In normal mice, the IEL $\gamma\delta$ + population represent 30-50%, depending upon the degree of antigenic stimulation and the age of the animal. However, differences have been found in other species, specifically chickens and humans (Bucy 1988, Groh 1989). There is no evidence for a general epithelial tropism in man, and these cells are not predominant in other epithelia such as skin, respiratory tract, or the reproductive organs where their proportion is similar to that found in other lymphoid compartments (<5% of the total T cell number).

In normal human intestine, TCR $\gamma\delta$ + cells are segregated

preferentially in the epithelium, as compared with TCR $\gamma\delta$ + cells from other localizations, but the majority of IEL and lamina propria lymphocytes express the TCR $\alpha\beta$ + (Groh 1989, Tredjosiewicz 1989, Spencer 1989a, Haltensen 1989, Bucy 1990, Jarry 1990, Vroom 1991). The proportion of TCR $\gamma\delta$ + detected is dependent upon the specificity of the mAb used. Studies using the mAb δ TCS-1, that identifies a subset positive for the V δ 1/J δ 1 gene segments and mucosal-specific, showed that 2% of the total IEL population are TCR $\gamma\delta$ + (Spencer 1989a). Higher proportions have been found using TCR $\gamma\delta$ framework antibodies, up to 15% of the total CD3+ cells in small and large bowel epithelium (Groh 1989, Bucy 1989, Haltensen 1989, Fukushima 1991), mainly CD4-CD8- and CD45RO memory cells (Haltensen 1990). This is contradicted by the finding of higher numbers in small bowel and colonic mucosa (Tredjosiewicz 1989, (Ullrich 1991)), and by the finding of a predominant expression of CD8 antigens by TCR $\gamma\delta$ + V δ 1+ IELs (Deusch 1991, Lynch 1993).

Gut epithelium as a site of T cell maturation

Based on evidence from animal studies, gut epithelium might have a role in extrathymic T cell development by promoting the differentiation of immature cells to CD3+ CD8 α + T cells, without thymus involvement (Rocha 1993).

Foetal TCR gene rearrangements may occur earlier in some IEL than in thymic lymphocytes (Carding 1990). In experiments with athymic mice, it has been observed that CD3+ CD8 $\alpha\alpha$ monodimeric cells repopulate the epithelium (Guy-Grand 1991); and in animals with severe combined immuno-deficiency (scid/scid), where Ig and TCR gene rearrangements should not be possible, the intestinal epithelium contains immature cells expressing the CD8 α chain (Croitoru 1990). An IEL homodimeric CD8 $\alpha\alpha$ population has been recently identified in single cell suspensions from human small intestinal biopsies (Lynch 1993), and that may suggest the existence of a thymus-independent differentiation process in humans (Jarry 1990).

TCR $\gamma\delta$ + cells appear during intrathymic development and represent the CD4-CD8- population in peripheral blood. Most $\alpha\beta$ + IEL are CD4-CD8+; whereas 50-70% of $\gamma\delta$ + IEL are CD3+ CD4-CD8-, and the remainder express only the α chain of the heterodimer (CD8 $\alpha+\beta$ -) (Brandtzaeg 1989b, Jarry 1990, Cerf-Bensussan & Guy-grand 1992). The expression of the second CD8 α molecule by $\gamma\delta$ + IELs seems to be secondary to the migration of precursors to the intestinal mucosa, since the $\gamma\delta$ + CD8+ (β -) subset is absent from peripheral blood. In mice, the co-expression of TCR $\gamma\delta$ + and the CD8 $\alpha\alpha$ homodimer characterized cells undergoing extrathymic development and has only been

found in epithelium, apparently induced by local factors (Janeway 1988, Jarry 1990, Fukushima 1991).

In mice, an extra-thymic differentiation of T cells may explain some of the features of $\gamma\delta$ IEL, such as selective localization in epithelia, preferential usage of a particular V gene region at different anatomical sites, and the existence of very limited repertoires in some epithelia (Bandeira 1991, Rocha 1993). This is also supported by the finding in murine enterocytes of a class I-like (CD1) molecule, which is normally expressed by thymocytes and dendritic cells. Its human equivalent CD1b, may function as a restriction element in the enterocyte/IEL interaction by an alternative pathway of T cell activation (Strominger 1989, Guy-Grand & Vassalli 1993).

It has been suggested that a preferential localization of TCR $\gamma\delta$ cells in intestinal epithelium may be explained by either a developmentally regulated process after productive rearrangements in bone marrow or elsewhere and then specific migration to the epithelium, or by a peripheral antigen-driven expansion where precursors migrate at random, rearrange in situ, and only undergo maturation and differentiation in the epithelium (Spencer 1989a, Kluin 1991). There is probably a mixture of both, a regulated seeding of precursors from thymic and

extrathymic origins, and a peripheral expansion under the local microenvironment that shapes the functional IEL $\gamma\delta$ repertoire (Kluin 1991).

Possible role of TCR $\gamma\delta$ + cells in intestine.

Intestinal TCR $\gamma\delta$ + cells may function as suppressor/cytotoxic cells involved in the control of inflammatory responses and/or regulation of mucosal responses to dietary antigens (Barrett 1993, Fujihashi 1992). These cells may produce cytokines in vivo, which may affect B cell differentiation and immunoglobulin synthesis, but also other mucosal functions (Brandtzaeg 1988, Morita 1991, Christmas 1991, Tagushi 1991). Of interest is the unusual finding that both IL-5 and IFN γ can be produced by the same IEL population (Taguchi 1991). These TCR $\gamma\delta$ + cells might represent a population of contra-suppressor cells that restore antibody responses in mice previously orally tolerized with specific antigens in vivo (Fujihashi 1989, 1992). This CD4-CD8- and potentially immature $\gamma\delta$ + IEL population may expand in situations of tissue damage and increased cell turnover. Precursor double-negative IELs have been involved in spontaneous LAK activity (Ebert 1989, 1990).

TCR $\gamma\delta$ + cells in coeliac disease

Coeliac disease and dermatitis herpetiformis (DH) are the only human diseases in which a consistent TCR $\gamma\delta$ + T cell increase has been observed in the small intestinal mucosa (Haltensen 1989, Spencer 1989a, 1991; Rust 1992; Savilahti 1990, 1992; Kluin 1991, Tredjosiewicz 1991, Vecchi 1992). The increased proportion of CD3+ in epithelium coincides with the previously reported increase (2 to 30%) of the CD3+ CD4-CD8- cell population (Verkasalo 1990). These $\gamma\delta$ + IELs express preferentially the V δ 1/J δ 1 phenotype (recognized by the mAb δ TCS-1) and remain increased in untreated and treated coeliacs with normal mucosal morphology (Spencer 1989a, Haltensen 1989, Trejdosiewicz 1989, Savilahti 1990, Kluin 1991, Deusch 1991). However, some of these cells express neither V δ 1 nor V δ 2 gene products (Spencer 1989a, DeLibero 1993), and this has also been observed in two suspected coeliac cases with morphologically normal biopsies (Kluin 1991).

In coeliac disease, it has been suggested that $\gamma\delta$ + IELs may function as cytotoxic cells activated either by gluten, or indirectly by cytokines released by gluten-specific TCR $\alpha\beta$ + cells, leading to epithelial damage or the destruction of already damaged cells (Brandtzaeg 1989a, 1991; Viney 1990, Kluin 1991, Russell 1991). However, TCR $\gamma\delta$ + cells are not increased in other

intestinal diseases associated with villous atrophy and crypt hyperplasia such as tropical sprue, severe intestinal food allergy, Crohn's disease or autoimmune enteropathy (Cuenod 1990, Fukushima 1991, Tredjosiewicz 1991, Cuvelier 1992, MacDonald 1992). An exception may be cow's milk intolerance in children (Spencer 1991), though these children may be latent coeliac patients.

The surface epithelium of coeliac mucosa has an enhanced expression of hsp, and this may represent the association between $\gamma\delta$ cells and reactivity to self components. Constant exposure to autologous hsp-65 presented by macrophages may lead to continuous lymphokine production which causes persistence of the lesion, as occurs in other situations (Soderstrom 1990). However, in coeliac disease, epithelial damage and release of hsp is not sufficient to increase IEL $\gamma\delta^+$ numbers (Harvey 1991, Kluin 1991). It has been also proposed that in addition to the presence of hsp, bacterial superantigens might induce segregation and accumulation of TCR $\gamma\delta$ cells within the gut in genetically predisposed individuals (Kutlu 1993).

High $\gamma\delta^+$ IEL counts, and dietary and mucosal status

The finding of high numbers of $\gamma\delta^+$ IELs has been found to be a constant feature in both coeliac disease and

dermatitis herpetiformis. This is not related to either the degree of mucosal damage or the time on a GFD (Spencer 1989a, 1991; Savilahti 1990, Viney 1990, Kutlu 1993). High counts of $\gamma\delta$ + IEL have also been reported in non-coeliac patients and in HLA-matched relatives of coeliacs (Groh 1989, Haltensen 1989, Kluin 1991). In contrast, other morphological, enzymatic and immunological abnormalities in small bowel intestine are reversed by diet, this includes the density of $\alpha\beta$ + IELs.

The association found between the $\gamma\delta$ + IEL expansion and the coeliac disease related HLA-DQA1/DQB1 haplotype in healthy first-degree relatives of coeliac patients (Holm 1992, 1993b) suggests the existence of an inherited immune abnormality related to the expression of this haplotype (eg a state of hypersensitivity to gliadin), and manifested by the expansion of TCR $\gamma\delta$ + cells in intestine (Maki 1991b, Savilahti 1992, Holm 1992, 1993b), though other unknown genetic factors cannot be excluded. In mice, the expansion of the $\gamma\delta$ + IEL subset may be related to one MHC-class II haplotype (Lefrancois 1990). Alternatively, $\gamma\delta$ + IELs might undergo polyclonal expansion controlled by local regulatory factors triggered by gluten, to which predisposed individuals may be specially susceptible. Cytokines may be such a factor which modulate antigen presentation by the induction of MHC-class II expression (Kluin 1991, Deusch 1991, Marsh

1992, DeLibero 1993, Kutlu 1993).

TCR $\gamma\delta$ + cells have very limited diversity, and thus limited capacity for antigen recognition (Allison & Havran 1991, Rust 1992). However, phenotypic and genotypic analysis of $\gamma\delta$ T cells in coeliac disease have also shown also heterogeneity rather than a monoclonal expansion of a particular subset. It has been suggested that more than one antigenic determinant and/or stimulus may be responsible for the expansion of this population (Tredjosiewicz 1991, Rust 1992, DeLibero 1993). Moreover, TCR $\gamma\delta$ + cells can be found in human foetal intestine, which suggests that gluten is not necessary for their expansion (Spencer 1989b, Haltensen 1989). The fact that a high density of $\gamma\delta$ IELs was found in a latent coeliac patient before the development of enteropathy (Savilahti 1992), and in treated patients after years of gluten restriction (Kutlu 1993), as well as the high $\gamma\delta$ IEL count that I found in several non-coeliac patients, argue against their involvement in mucosal damage.

After gluten challenge experiments, it is known that TCR $\alpha\beta$ + cells infiltrate the epithelium in a dose-dependent manner (Marsh 1980, Leigh 1985, Loft 1989), whereas the TCR $\gamma\delta$ + subset does not change significantly. Before this infiltration is observed, the lamina propria is occupied by neutrophils and other mononuclear cells, followed

immediately by an enhanced expression of the adhesion molecules ELAM-1 and VCAM-1 (Sturgess 1990, Ensari 1993). The gluten-sensitised population is formed by CD8+ TCR $\alpha\beta$ + IELs, which in untreated coeliacs express the heterodimer CD8 $\alpha\beta$, therefore these cells may be dependent on the thymus and primed in Peyer's patches by antigens (Guy-Grand 1991, Kutlu 1993).

Based on the differential expression of the nuclear proliferation marker recognized by the mAb Ki-67 in epithelium, and the p55 α chain of the IL-2R (CD25) in the lamina propria, it has been suggested that gluten induces a non-proliferative activation of lamina propria CD4+ cells, which may synthesize cytokines, and induces proliferation of CD8+ IEL of both phenotypes, TCR $\alpha\beta$ and $\gamma\delta$ (Haltensen & Brandtzaeg 1993). These cells undergo a polyclonal expansion which may be independent of the antigen but dependent on IL-2 produced by activated lamina propria CD4+ cells, and it may also explain the heterogeneity of the increased IEL population, in relationship to gluten specificity.

D. T CELL-MEDIATED IMMUNE RESPONSES IN HUMAN INTESTINE

Delayed type hypersensitivity reactions and mucosal morphology

The coeliac lesion reflects a T cell-mediated delayed type hypersensitivity (DTH) reaction to gluten mediated by lymphokines and other cells involved in non-specific inflammatory processes. There is evidence of T cell involvement and lymphokine secretion by cultured jejunal biopsies after gluten stimulation (MacDonald & Ferguson 1976). The lesion is similar to the intestinal graft-vs-host disease in animal models (Mowat & Ferguson 1982, Guy-Grand & Vassalli 1986, Mowat 1987), manifested by villous atrophy, crypt hyperplasia, increased IEL infiltration, and increased HLA-DR expression by epithelial cells. Treatment with anti-TNF α or anti-IFN γ antibodies prevents the intestinal infiltrate and the mucosal damage in these animals (Piguet 1987, Mowat 1989).

Studies of cell kinetics in flat mucosa of the human small intestine showed that crypt hyperplasia is a primary event in the development of the coeliac lesion

(Wright 1973). Experiments of in-vitro culture of fetal explants, have also shown that T cell activation is associated with crypt cell hyperplasia even before any mucosal damage, and villous atrophy is the subsequent effect of crypt hyperplasia. The enteropathy can develop without a cytotoxic response to epithelial cells, and it is mediated by soluble factors release by activated lamina propria CD4+ cells that affect the structure and function of the small intestine (Griffiths 1988, MacDonald & Spencer 1988, Monk 1988, Ferreira 1990, MacDonald 1992).

The initial immunopathological event in gluten challenge studies is a migration of lymphocytes into the small bowel epithelium (Marsh 1980, Brandtzaeg 1989a). This may be a manifestation of T cell activation which, together with the degree of villous atrophy is related to gluten in a dose-dependent manner (Leigh 1985). In coeliac mucosa, gluten challenge leads to the accumulation of both phenotypes, lamina propria CD3+ CD4+ and epithelial CD3+ CD8+ CD45RO-memory or primed cells (recognized by the mAb UCHL-1). UCHL-1 is mainly expressed by TCR $\alpha\beta$ + cells, but a higher number of $\gamma\delta$ + cells also have this memory marker (Harvey 1989, Brandtzaeg 1989b, Halstensen 1990, Halstensen & Brandtzaeg 1991).



Current hypothesis of the coeliac lesion

The coeliac lesion is the result of an aberrant HLA-class II restricted immune response to gluten, and mononuclear cell infiltration (Sollid 1989, O'Farrelly & Gallagher 1992, Lundin 1993). Lamina propria CD4+ cells seem to play a central role in the development of the gluten-sensitive lesion. These cells are activated as confirmed by the presence of CD25+ cells amongst the CD4+/CD45RO+ cell population, and also CD3- cells (probably macrophages), followed by the expression of the Ki-67 proliferative antigen by both CD8+ $\alpha\beta$ + and $\gamma\delta$ + cells in epithelium, and other changes in mucosal structure (Marsh 1992, MacDonald 1992, Haltensen 1992b, Haltensen & Brandtzaeg 1993). MHC-class II positive macrophages might present antigen to CD4+ CD45RO+ cells leading to cytokine production and a cascade of immuno-regulatory effects (Marsh 1992). However, the phenotype, cytokine profile and function of the potential gluten-responding T cells, are not yet known.

Gluten-reactive T cells have been isolated from the intestine of coeliac patients. Most of these cells seem to be restricted by the coeliac-associated HLA-DQ α/β heterodimer (Lundin 1993). These HLA-class II region alleles are associated the susceptibility to coeliac disease, but it is not yet known whether this association

operates (Kagnoff 1992), by involvement of HLA-DQ molecules in the "selection" of the T cell repertoire (Altmann 1991), or by local events in mucosa, eg HLA-DQ restricted antigen presentation to T cells (Lundin 1993).

Evidence of immunologically-mediated mucosal damage

Activation of intestinal T lymphocytes is related to the presence of CD25+ cells and the increased expression of HLA-DR antigens by epithelial cells. However, early reports showed lymphoid cell infiltration but failed to detect other signs of activation, particularly CD25+ cells (Selby 1983, Kelly 1987). More recently, a high expression of HLA-class II molecules in epithelium, and CD25 antigens in the lamina propria, has been reported (MacDonald & Spencer 1988, Haltensen 1992b). There is evidence that IELs can modulate the HLA-DR expression by enterocytes, by IFN γ production (Cerf-Bensussan 1984, Scott 1987, Sturgess 1992). Interferon- γ may also induce epithelial SC expression (Sollid 1987, Kvale 1988b), inhibition of epithelial cell proliferation, and increased epithelial permeability (Madara & Stafford 1989).

T cell activation may also be associated with other changes in lamina propria, such as infiltration of neutrophils, basophils and MMC (Dhesi 1984, Marsh & Hinde

1985); activation and degranulation of eosinophils, MMC and neutrophils (Hallgren 1989, Horvath 1989, Colombel 1992, Desreumaux 1992, Talley 1992); presence of prostaglandins and other inflammatory mediators (Lavo 1990, Branski 1992), and activation of complement with subepithelial deposits of the terminal C complex (Haltensen 1992a, Scott 1992). Lymphocyte activation by gluten fraction III led to the expression of IL-2R (Penttila 1990), and soluble IL-2R levels have been found increased in serum from adults and children with active coeliac disease (Crabtree 1989b, Blanco 1992), which are normalized after gluten restriction.

T cells express the CD25 marker soon after activation by antigen (Reed 1986). In-vitro polyclonal T cell activation in human mucosa has shown that CD25 is expressed only by lamina propria T cells and macrophages (MacDonald & Spencer 1988, MacDonald 1990a). In the epithelium, there is an IEL infiltration, with increased epithelial cell kinetics and HLA-DR expression, but these cells are CD25 negative. In contrast, epithelial CD25+ cells have been reported in chronic rejection of a jejunal transplant (Cerf-Bensussan 1990). This in-vitro enteropathy is similar to the active coeliac lesion with a differential expression of the proliferative Ki-67 antigen by epithelial cells, and the non-proliferative CD25 marker by lamina propria CD4+ cells, which may be

associated with lymphokine synthesis (Haltensen & Brandtzaeg 1993). These events may explain the difficult isolation of gluten-specific T cells in mucosa, where IELs may be induced to proliferate in an antigen-independent (but probably IL-2 dependent) manner, and secondary to lamina propria CD4+ cell activation.

In a recent study, signs of inflammation detected by HLA-DR antigen expression and presence of CD25+ cells, have been shown in morphologically normal biopsies from first degree relatives of coeliac patients (Holm 1993a,b). These patients are "potential" coeliacs with a high number of $\gamma\delta$ + IELs, which correlates with the disease-associated genetic markers. They may eventually develop a gluten-sensitive enteropathy, and this may be monitored by the measurement of ARA/AEm serum antibody titers.

Patterns of cytokine production by T cell subsets

Studies in mice have shown that CD4+ cells can be subdivided into two subsets according to the pattern of cytokine synthesis (Mossman & Coffman 1989), and these subsets have different helper activity on immunoglobulin synthesis by B cells, and cytolytic potential. In humans, similar T cell clones may exist in both healthy individuals and disease patients, with different non-overlapping cytokine profiles (Salgame 1991, Del Prete

1991). These mature T cell clones may be differentially regulated by different antigen structures, populations of antigen-presenting cells, and conditions of immunization. Macrophage stimulation leads to proliferation of Th1 clones, whereas B cells stimulate preferentially Th2 clones (Gajenski 1991).

Th1 subsets produce mainly IL-2 and IFN γ . They are involved in cell-mediated immune responses, cytotoxicity, delayed-type hypersensitivity (DTH) reactions. They also mediate polyclonal Ig synthesis (IFN γ induces IgA2 and IgM. Kett 1990), when stimulated with high amounts of antigens, and inhibit the Th2 cell function (IFN γ blocks IL-4) under certain conditions.

Th2 subsets produce IL-4, IL-5, IL-6, IL-10; and are involved in IgM, IgG, IgE and IgA synthesis (specially in mucosa), and other non-specific inflammatory processes with MMC and eosinophils. Antigen absorption through Peyer's patches (eg oral immunization with cholera toxin) induces proliferation of Th2 clones. IL-10 blocks the in-vitro cytokine synthesis by Th1 clones, acting on the antigen-presenting function of macrophages (Fiorentino 1991). There is another group (**Th0**) of probable memory cells and unrestricted cytokine profile: IL-2, IFN- γ , IL-4 and IL-5. A high number of mucosal lymphocytes in mice produce IFN γ and IL-5 in vitro (Tagushi 1990).

Oral administration of soluble antigens might induce tolerance rather than a mucosal immune response, by preferential epithelial uptake of antigen, activation of lamina propria CD4+ cells by macrophages, and subsequent production of inhibitory cytokines (IL-4, TGF β , IL-10) with suppressive effect on the induction of immune responses.

Cytokines as mediators of mucosal damage

T cell activation leads to morphological and functional changes in the small bowel mucosa, and it is likely that cytokines can act as mediators. Cytokines may be involved in different activities, such as IgA production; clonal expansion, activation and differentiation of lymphoid cells; immuno-regulatory mechanisms of oral tolerance; modulation of the endothelial receptor function; epithelial cell physiology and stimulation of epithelial crypt cell growth; and healing mechanisms in mucosa (MacDonald & Spencer 1988, Vidrich 1991, Wershill 1992).

- Interferon-gamma

IFN γ may modulate the epithelial permeability by acting on epithelial cell tight junctions (Madara & Stafford 1989), antigen presentation, and mucus secretion. IFN γ

up-regulates the epithelial expression of a variety of cellular receptors, including MHC-class II antigens and secretory component (Kvale 1988b). Intra-epithelial CD3+ CD8+ $\alpha\beta$ + cells, in turn, may produce great amounts of IFN γ (Scott 1987, Brandtzaeg 1989a, Sturgess 1992). It has also been shown that IFN γ produced by activated T cells may kill colonic epithelial cells (Deem 1991). Original studies at single cell level (MacDonald 1990b), and by immunohistochemistry (Al-Darwood 1992) in coeliac disease failed to detect IFN γ -secreting cells. However, others have found a correlation between the number of IFN- γ producing cells, increased in untreated coeliacs, and the degree of villous atrophy (Kontakou 1993).

- Interleukin-2 receptor

Antibodies to IL-2R identify a low affinity α chain of the IL-2 receptor (Tac antigen), which is essential for T cell activation and proliferation. High serum levels of IL-2R have been used as a marker of disease activity in autoimmune and malignant diseases (Rubin 1985, Manoussakis 1989) though IL-2R seems to be released into the circulation asynchronously with immune activation. Interleukin-2 receptor is mainly produced by helper T cells in direct relationship to IL-2 production, and in serum it may have an in-vivo immunoregulatory function, though this is still unknown (Rubin 1985, Mueller 1990).

Adults and children with active coeliac disease, have increased levels of soluble IL-2R in serum, which decrease after treatment (Crabtree 1989b, Blanco 1992). Serum levels correlate with soluble CD4 antigens, but not with serum anti-gliadin antibodies (Blanco 1992). Activation of peripheral blood lymphocytes by gluten fraction III has been measured by detection of soluble IL-2R levels in serum (Penttila 1990), however, though its measurement may be an index of immune activation, its cellular origin is unknown (MacDonald 1993).

- Tumor necrosis factor-alpha

TNF α is mainly produced by macrophages, but also by NK cells and T cells. It has a short life in plasma and other body fluids, and is sometimes undetectable despite local production by inflamed mucosa. TNF α is regulated differently in each tissue, and there is no correlation between compartments, or between cytokine levels and TNF-secreting cells (MacDonald 1990b, Meager 1990). TNF α affects epithelial function and causes acute intestinal injury, probably by a direct effect on the microcirculation, as observed by intravenous injection of TNF α in animals, with endothelial and epithelial cell damage, neutrophil accumulation, and necrosis of villi (Remick 1987). The effect on microvascular permeability and blood flow may be mediated by stimulation of nitric

oxide production (Liew & Cox 1991), which is also produced by LPS or IFN γ activated macrophages (Stuehr & Marletta 1987). It has been shown that murine Paneth cells may also express mRNA for TNF α (Keshaw 1990).

Proinflammatory cytokines, specially TNF α and IFN γ , but also IL-1 and IL-6 are not detectable unless stimulation occurs. In the intestinal mucosa, these cytokines are involved in T and B cell activation, regulation of the expression of homing receptors, and synthesis of components necessary for IgA transport. In coeliac disease, IL-1 and IL-3 may affect the epithelium by stimulation of prostaglandin E2 production (Branski 1992). Subepithelial fibroblasts support the villous architecture but they may also influence the response to inflammatory mediators and cytokines (Berschneider & Powell 1992). Locally produced TNF α and IL-1 are involved in neutrophil migration, by inducing the expression of adhesion molecules and chemotactic factors (Kishimoto 1991). Enterocytes may express the intra-cellular adhesion molecule-1 (ICAM-1), and lymphocyte function-associated antigen-3 (LFA-3), but only the first is up-regulated by IFN γ and IL-1 β (Kaiselian 1991), and by TNF α and IL-6 (Kvale 1992).

Activated MMC may produce TNF- α , prostaglandins and leukotrienes, but they may also play a role in fibrotic

processes via TGF β production (Pennington 1992). Isolated eosinophils express mRNA for TGF β (Wong 1991), IL-3 (Kita 1991), and IL-5 in coeliac disease (Desreumaux 1992). TGF β may be synthesized by lymphocytes, macrophages and epithelial cells; it regulates the maturation of intestinal epithelial cells and enhances IL-6 production in epithelial cell lines (McGee 1992). Both IL-6 and TNF- α producing cells have been found increased in untreated coeliac patients, but also there is an increase in the number of lamina propria macrophages that might be involved in cytokine synthesis (Przemioslo 1993). In experimental colitis in rats, colonic epithelial cells express IL-1 β mRNA (Radema 1991).

Expression of HLA-class II antigens in intestine

The polymorphism of the HLA-class II proteins ($\alpha\beta$ heterodimers) determine a spectrum of antigen binding affinities in each individual (Brandtzaeg 1991). In coeliac disease, the immune responsiveness to gluten may be modulated by selection of the TCR repertoire in thymus or by HLA-restricted antigen presentation at local level (eg HLA-DR $^{+}$ enterocytes, or lamina propria HLA-DQ $^{+}$ macrophages). HLA-class II antigens are needed for monocytes, macrophages and B cells to become antigen-presenting cells (Ziegler & Unanue 1981), and also in normal intestinal enterocytes, though the latter

selectively stimulate nonspecific suppressor cells (Bland & Warren 1986, Mayer & Shlien 1987). It is also known that HLA molecules neither bind gliadin in a lectin-like fashion, nor have antigenic cross-reactivity with gliadin (Gallagher 1988).

HLA-DR is normally expressed by human villous epithelial cells, particularly at the tip of the villi, and decreases towards the crypt, where it is undetectable (Scott 1980b, Kelly 1988, Montgomery 1988). HLA-DR expression by crypt epithelial cells may be used as a marker of local DTH reaction (Ferguson 1987a, Fais 1992). In coeliac and other inflammatory diseases, the HLA-DR expression by villous and crypt epithelium is gluten dependent, and may be secondary to the lymphocyte infiltration, and IFN γ synthesis (Scott 1981, 1987; Cerf-Bensussan 1984, Ciclitira 1986a, Arnaud-Battandier 1986, Spencer 1986, Marley 1987, Sturgess 1992). Because HLA-DR+ cells can be found in uninvolved areas, it has been suggested that these cells may represent an early change in inflammation, or a group of immunoregulatory cells inducing secondary inflammatory responses (Mayer 1991).

HLA-DP expression has a similar distribution, but a lower intensity than DR expression in villous epithelium or expression in other cells (Marley 1987, Mayer 1991). HLA-DQ antigens are absent in normal intestine, and

positive cells can only be found in lamina propria (Sarles 1987, Schweizer 1991). In active coeliac disease HLA-DQ expression may be found in the lamina propria, and gluten specific HLA-DQ restricted T cells have been isolated from coeliac jejunal biopsies (Lundin 1990, 1993).

Expression of the CD25 activation marker in intestine

The CD25 marker is expressed soon after antigen stimulation (Reed 1986). Experiments of polyclonal T cell activation of human mucosa in vitro, led to the expression of CD25 markers by lamina propria T cells and macrophage-like cells (MacDonald & Spencer 1988, MacDonald 1990). In epithelium, this activation was manifested by IEL infiltration and HLA-DR expression by enterocytes. The degree of tissue destruction has been associated with high numbers of large CD3-CD25+ cells, probably macrophages, in studies of fetal tissue culture (Lionetti 1993), and this destruction may be secondary to the T cell activation in lamina propria and IL-2 and IFN γ production, which are potent activating factors for macrophages (Belosevic 1988, Cox 1990). In-vivo similarities are also found in the mucosa of untreated coeliacs, where a differential expression of activation markers is observed, the Ki-67 proliferative marker is expressed by enterocytes, and the CD25 marker by lamina

propria cells (Haltensen & Brandtzaeg 1993). This might suggest that the IEL population is induced to proliferate in an antigen-independent manner, explaining the absence of gluten-specific T cells, but probably dependent on IL-2 production, and secondary to LP CD4+ cell activation.

Autoreactive CD5+ B cells

It has been suggested that polyreactive, low affinity IgM antibodies, produced by CD5+ B cells that recognize self determinants, may represent a first line of defence against infectious agents (Casali & Notkins 1989). In rheumatoid arthritis, there is an expansion of the T-cell population bearing the $\gamma\delta$ TCR, and of B cells expressing the CD5+ marker in peripheral blood (Plater-Zyberk 1989), but they are not related to the severity of the disease activity. It may be hypothesized that a genetically determined immunoregulatory abnormality may lead to the development of a "fetal cell type" population (CD5+ B cells, $\gamma\delta$ + T cells) involved in the generation of an enhanced reactivity to common bacterial antigens which are antigenically conserved between species (Brennan 1989). Furthermore, these cells may not respond to local factors to avoid clonal expansion which would lead to excessive auto-reactivity and formation of immune-complexes. Studies in mice have shown that treatment with anti-IL-10 from birth causes a reduction of serum IgM

concentration and the elimination of CD5+ B cells, which have been postulated to be the principal source of circulating IgM antibodies (Hayakawa 1983).

In fetal life, HLA-DQ antigens might be involved in the elimination of autoreactive T cells, and some HLA-DQ phenotypes might allow these clones to survive (Altmann 1991). Moreover, HLA-DQ expression is absent from coeliac and non-coeliac epithelium, suggesting the existence of an epithelial defect for these antigens (Kelly 1988, Mayer 1991, Chapter XII).

SECTION TWO

MATERIALS AND METHODS

CHAPTER II

PATIENTS AND COLLECTION OF SPECIMENS

A. PATIENTS

This Gastro-Intestinal Unit provides a regional service for the clinical investigation of gastro-intestinal function. Patients were collected over a period of 30 months but selected by the availability of samples. All jejunal biopsies were taken for diagnostic purposes and in the course of a full range of investigations; or in the follow up of coeliac cases to assess their response to the dietary treatment.

Coeliac patients and controls (preliminary study)

Jejunal fluid and serum samples were collected on 80 occasions from 69 patients. There were 41 with coeliac disease (7 studied twice), 23 women and 18 men (age range of 15-78 years, median 42); and 28 control patients, 14 women and 14 men (age range of 14-75 years, median 35), referred for diagnostic jejunal biopsy, eventually found to be normal, with no other significant pathology, and a final diagnosis of functional bowel disease.

Of the coeliacs, 26 were untreated and their jejunal biopsy showed subtotal or severe partial villous atrophy. Eleven of these, and a further 15 treated coeliacs (all with previous diagnostic biopsies) were re-biopsied while on a GFD. Median period on GFD was 3 years (range 3 months-17 years). Eleven had entirely normal jejunal histology (all of these had been taking a GFD for at least 2 years), and 11 had minor histological changes, eg increased IEL counts (most patients in this group had been taking a GFD for less than one year). Four further patients whom it had been our intention to study showed no histological improvement on a GFD. We attributed this to non-compliance and thus excluded them; thus, data was collected from 76 studies.

Patient series A, coeliac and non-coeliacs

During a 16-month period, a total of 151 patients attended for per-oral jejunal biopsy. Adequate samples of jejunal fluid and small bowel mucosa (not suitable for frozen sections) were collected at the same time from 140 patients, 91 women and 49 men, with an age range of 13-83 years (median 37). Serum samples were collected from 137 of these patients. All specimens from this prospective cohort study were assessed blinded, therefore 22 of the series were coeliac patients having follow-up biopsies to

assess their response to a GFD. A decision as to the final diagnosis for the remaining 118 cases was made 2-3 months after the biopsy procedure, from examination of the case records, and without knowledge of the results of secretion or serum antibody tests. Of the 98 patients with a final diagnosis other than coeliac disease or DH, 93 were reported to have a histologically normal jejunal biopsy and 5 had minor histological abnormalities (short villi in two, excess inflammatory cells in three).

Details of the patients, their diets, final diagnoses, and biopsy histology, are given in Table II.1. Diagnostic groups of series A:

Coeliac disease (COELIAC) and Dermatitis Herpetiformis (DH) A total of 36 biopsies were obtained from 33 coeliac patients (3 of them were biopsied twice). There were 14 new cases of coeliac disease, and 22 biopsies from treated patients, the majority with less than one year of GFD, with improved but still unequivocally abnormal histology. Five of the six DH patients were newly diagnosed, and one was on a GFD.

Not coeliac (NOT COE) There were three patients in whom a diagnosis of coeliac disease had previously been made on inadequate criteria (no biopsy or normal biopsy); in the final analysis they have all been classified as

TABLE II.1. FINAL CLASSIFICATION OF PATIENTS BY DIAGNOSIS AND BY JEJUNAL BIOPSY HISTOLOGY. SERIES A.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD	HISTOLOGY			PTVA
					NORMAL	MINOR CHANGES		
COELIAC Untreated	14	39 (19-82)	8/6	14			14	
Treated	22	39 (14-82)	15/7		22	4	2	16
DH	6	44 (30-60)	4/2	1	5	1	2	3
NOT-COE	3	27 (14-38)	2/1	1	2	3		
IBD	7	29 (18-41)	4/3	7		5	2	
IDIO DIA	23	36 (15-67)	11/12	23				23
ORAL ULC	6	37 (19-80)	5/1	6				6
MISC	23	46 (16-76)	15/8	23				23
NUT DEF	8	49 (23-69)	5/3	8		7	1	
IBS	13	35 (20-45)	10/3	13		12	1	
NAD	15	34 (13-57)	11/4	15		14	1	

DH= dermatitis herpetiformis

NOT-COE= previous diagnosis on inadequate criteria

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= high IEL counts, inflammatory cell infiltrate

PTVA= severe partial, or total villus atrophy

healthy.

Inflammatory bowel disease (IBD) Four patients had Crohn's disease and three had ulcerative colitis.

Idiopathic diarrhoea (IDIO DIA) These 23 patients had chronic, watery diarrhoea, and no firm diagnosis was made even after full investigation by a consultant gastroenterologist. One was also IgA deficient.

Oral ulceration (ORAL ULC) These were patients with severe aphthous ulceration or oral candidiasis (HIV negative), referred for malabsorption work-up by Dental surgeons and in whom no evidence of intestinal disease emerged.

Miscellaneous GI disease (MISC) There were 23 patients with various significant organic GI diseases, including diverticular disease, small bowel bacterial colonization, colonic polyps, giardiasis, colorectal cancers, radiation entero-colitis, drug-induced diarrhoea, alcoholic cirrhosis.

Nutritional deficiencies (NUT DEF) Seven patients had presented with anaemia or weight loss, ultimately attributed to poor diet.

Irritable bowel syndrome (IBS) These were patients with abdominal pain, bloating and variable bowel habit with no identifiable organic cause.

No abnormality detected (NAD) There were a few patients who had trivial symptoms or psychiatric disease, or had constitutional short stature.

Non-coeliac patients, series B

Similar studies were performed in a further cohort of 119 non-coeliac patients, from whom jejunal biopsy (not suitable for frozen sections), jejunal fluid, and serum specimens, were collected in the following 11-month period. These included 76 women and 43 men, with an age range of 15-79 years (median 36.5). A normal jejunal biopsy histology was found in 111 cases, and 8 showed minor histological changes (eg high IEL counts). Details of their diets, final diagnosis and jejunal biopsy histology are given in table II.2.

Non-coeliac patients, series C and D

Material suitable for the full range of immunological investigations, including frozen biopsy sections, was obtained from a further 77 non-coeliac patients (**series C**), 49 women and 28 men, age range of 17-77 years (median

TABLE II.2. FINAL CLASSIFICATION OF NON-COELIAC PATIENTS BY DIAGNOSIS AND BY JEJUNAL BIOPSY HISTOLOGY. SERIES B.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET		HISTOLOGY		
				NORMAL	GFD	NORMAL	MINOR CHANGES	PTVA
NOT COE	8	28.5 (20-63)	6/2	6	2	6	2	
IBD	14	45 (15-79)	7/7	14		14		
DIA IDIO	13	32 (20-67)	7/6	12	1	12	1	
ORAL ULC	12	25.5 (18-51)	9/3	12		11	1	
MISC	34	50.5 (14-77)	19/15	33	1	33	1	
NUT DEF	7	33 (26-45)	5/2	7		5	2	
IBS	18	35 (21-71)	13/5	18		18		
NAD	13	33 (15-55)	10/3	13		11	2	

NOT-COE= previous diagnosis on inadequate criteria

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities
(ie. high IEL counts, chronic inflammatory cell infiltrate)

PTVA= severe partial, or total villus atrophy

33). A decision as to the final diagnosis for these patients was made 2-3 months after taking the biopsy, from examination of the case records, and without knowledge of the results of immunological investigations. Details of the patients, their diets, final diagnosis, biopsy histology and sugar permeability test, are given in Table II.3. There was an additional diagnostic group, of first degree relatives (FH) of coeliacs, subsequently shown to be normal.

With the aim of validating the results and establishing reference values for $\tau\delta+$ IEL counts in frozen sections of small bowel intestine, **duodenal biopsies** from the distal second part of the duodenum were collected from a further 26 patients undergoing upper GI endoscopy (**series D**), none of whom had symptoms of small bowel disease. These included 17 women and 9 men; age range 16-87 (median 69.5); grouped by 6 different diagnoses: duodenal ulcer (4 cases), gastric ulcer (4), hiatus hernia (3), oesophagitis (4), other diseases (gallstones, portal hypertension, cancer), and no abnormality was detected in 8 cases.

As part of the technical appraisal of CD3+ and $\tau\delta+$ cell counts, frozen sections of jejunal biopsies from a group of coeliac patients (predicted to have high $\tau\delta+$ IEL counts) was also examined. Fifteen jejunal biopsies were

TABLE II.3. DEMOGRAPHIC DATA OF PATIENTS CLASSIFIED BY FINAL DIAGNOSIS AND JEJUNAL BIOPSY HISTOLOGY. SERIES C.

DIAGNOSIS	(n)	AGE mean (range)	SEX f/m	DIET		HISTOLOGY			PERMEABILITY	
				N	GFD	N	MINOR CHANGES	PTVA	N	Abn
NOT COE	4	30.6 (17-64)	2/2	2	2	3	1		4	
FH	4	26.8 (17-44)	4/-	3	1	4			4	
IBD	6	38.5 (23-65)	3/3	6		5	1		2	4
DIA IDIO	14	32.6 (21-70)	6/8	13	1	12	2		13	1
ORAL ULC	4	27 (19-52)	3/1	4		3	1		3	1
MISC	19	42.6 (19-77)	13/6	19		18	1		17	2
NUT DEF	4	35 (30-46)	4/-	3	1	4			4	
IBS	16	38 (21-71)	10/6	16		16			15	1
NAD	6	30 (21-52)	4/2	6		6			6	
COELIAC	15	42.7 (16-86)	12/3	2	13	5	2	8	7	8

NOT-COE= previous diagnosis on inadequate criteria

FH= relatives of coeliac patients

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

N= normal

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

collected from 14 coeliac patients, 11 women and 3 men, with an age range of 16-86 (median 37.5). Thirteen were already diagnosed, attending for follow-up biopsies (one of these had two biopsies, on a GFD and during a gluten challenge); only one was a newly presenting patient.

A total of 84 serum samples including all 77 non-coeliac patients from series C, and 7 other samples from previous series (5 women, 2 men; with 5 different diagnostic groups), were studied for anti-reticulin (ARA) and anti-endomysium (AEm) antibody titers (Dr Maki, Tampere, Finland). Serum samples from 6 coeliac patients (1 untreated, 1 on gluten challenge, 4 treated) were also included. (See Chapter VII for details).

Patients studied for total IEL counts

Biopsies from 171 patients, all with normal villous architecture, were assessed for IEL counts, 7 coeliacs on a GFD, 4 patients with DH, 44 non-coeliacs from **series A**, 39 non-coeliacs from **series B**. In total there were 40 CIA positive non-coeliac cases and 43 patients with a similar range of diagnoses but normal intestinal antibody patterns. Conventional IEL counts were performed in all 77 non-coeliac biopsies from **series C**, which have also CD3+ and epithelial $\gamma\delta$ + cell counts in cryostat sections, to allow comparisons between both counting

methods.

Patients studied for lamina propria plasma cell counts

Jejunal biopsies from patients included in Series A were studied for lamina propria IgA+ and IgM+ containing plasma cells, and MMC. From these, 12 were coeliacs, 6 newly diagnosed and 6 on a gluten-free diet (2 of them had normal biopsy histology), and 29 non-coeliac patients (26 showed a morphologically normal biopsy, and 4 minor histological changes. ie, high total IEL counts). See Chapter VIII for details.

Patients studied for the intestinal expression of HLA-DR, DQ and CD25 antigens

These included 32 non-coeliac patients, age range of 20-72 (median age: 31.5), 10 of these were patients diagnosed of having a functional disorder, and no abnormality was detected in 5 cases. These patients were grouped by their total IEL count, 16 with high counts, and 16 with normal counts. Sixteen coeliac patients, age range: 12-62 (median: 37) were also studied, 7 of them were untreated, one was undergoing a trial of gluten challenge, and 8 were treated. See Chapter IX for details.

Patients studied for TNF α and soluble IL-2R levels in jejunal fluids

Suitable specimens from 82 patients, 59 women and 23 men, included in series A and B, were studied for levels of tumour necrosis factor alpha (TNF α) and soluble IL-2R levels (Table II.4). These included 24 coeliac patients, 19 treated (8 had a normal biopsy, and 11 had either PVA or TVA) and 5 untreated; and 58 non-coeliacs, divided into a control group of 28 patients with a functional disorder or those in whom no abnormality was detected after investigation (all with normal biopsy histology), and a group of 30 patients with other organic GI diseases (27 with normal biopsy histology, 2 minimal changes, and 1 partial villous atrophy). Of these 83 patients, 37 had enough material to measure jejunal fluid IFN γ levels, including 9 coeliacs (2 were untreated, 7 on a GFD), 3 DH patients (1 untreated, 2 on a GFD); and 25 non-coeliac patients (3 IBD, 6 mouth ulcers, colonic cancer, etc), 10 of them described as controls (see above).

The different series of patients, and some of the studies performed in each group are represented in Figure II.1.

TABLE II.4. DEMOGRAPHIC DATA OF PATIENTS STUDIED FOR SOLUBLE IL-2R AND TNF α LEVELS IN JEJUNAL FLUID.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD	HISTOLOGY NORMAL MINOR PTVA CHANGES		
COELIAC Untreated	5	38 (15-75)	5/-	5			5
Treated	19	51 (15-76)	13/6	19	8	1	10
IBD	3	49 (45-54)	2/1	3	3		
DIA IDIO	6	32 (21-51)	5/1	6	4	2	
ORAL ULC	8	30 (18-52)	6/2	8	6	2	
MISC	11	47.5 (26-71)	6/5	11	11		
NUT DEF	2	30 (26-32)	2/-	2	2		
IBS	15	38 (21-71)	11/4	15	15		
NAD	13	36 (21-55)	9/4	13	11	2	

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

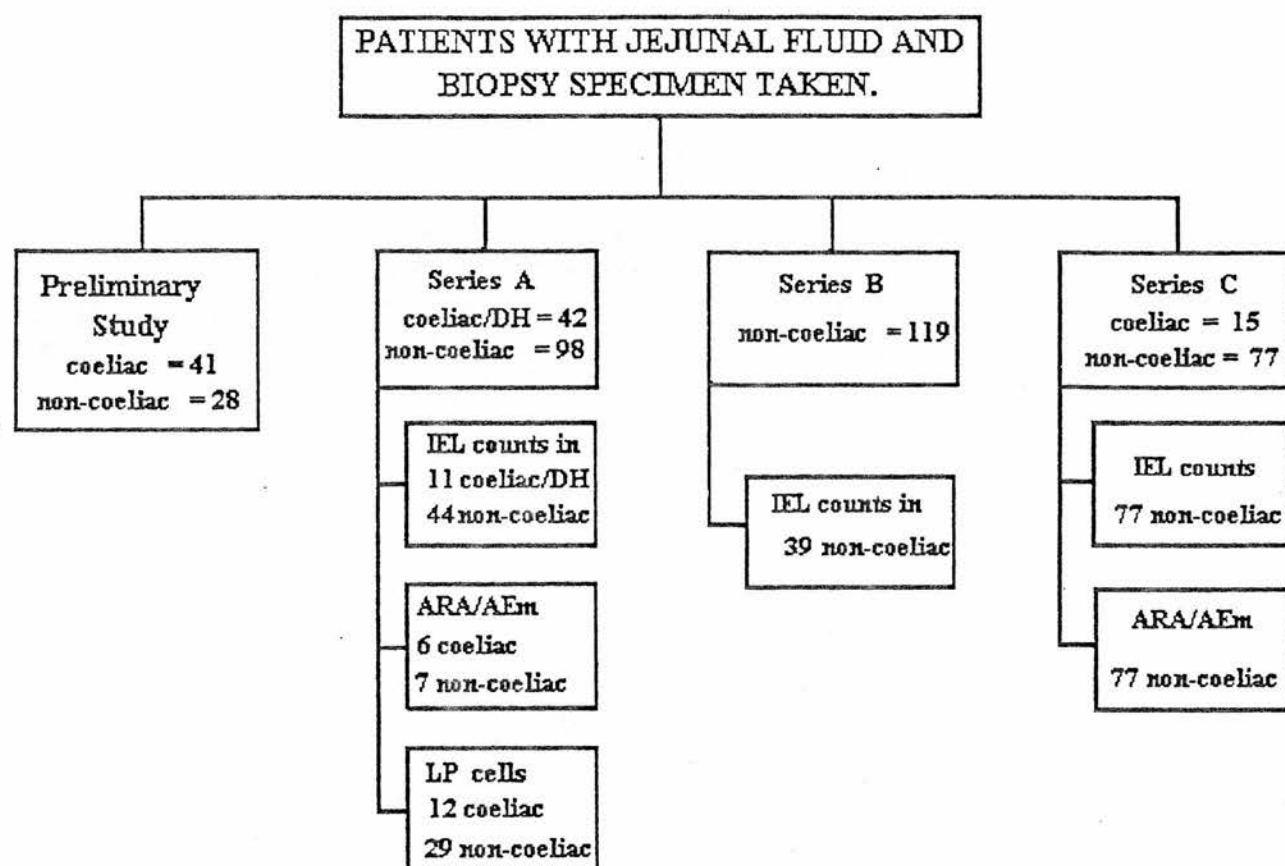


Figure II.1: Patients referred to the G.I. Unit because of possible small bowel disease, or in treated coeliac patients for follow up studies.

LP-cells= lamina propria plasma cells

B. COLLECTION OF SPECIMENS

Collection and processing of jejunal fluids

After an overnight fast, patients (referred for per-oral jejunal biopsy) swallowed a Watson biopsy capsule together with 15 mg of Metoclopramide. When the capsule had passed to the first loop of jejunum (assessed by X-ray screening), 1-2 ml intestinal fluid was drained by gravity through the tubing. The protease inhibitor phenylmethyl sulphonyl fluoride (PMSF), 20 ul/ml at a concentration of 0.1M, was immediately added. The capsule was then fired to obtain a jejunal mucosal biopsy. Jejunal fluid was transferred on ice to the laboratory and stored at -70°C within 10 minutes of collection. Venous blood samples were also taken, serum separated, aliquotted and stored at -70°C .

Collection of jejunal biopsies

Jejunal mucosal biopsies were formalin-fixed and sent to the diagnostic pathology laboratory, where a report was issued which included a detailed textual description and a codeable classification, ie. total villus atrophy (TVA), severe partial villus atrophy with crypt hyperplasia (PVA), minor non-specific abnormalities

(these included villus shortening without crypt hyperplasia, and general increase in inflammatory cell infiltrate) and morphologically normal.

Collection of biopsies for frozen sections (Series C, D)

Jejunal mucosal biopsies were orientated with the aid of a dissecting microscope and divided into two pieces. One was formalin-fixed and sent to the diagnostic pathology laboratory (see above) for subjective examination by a consultant pathologist. The other piece was embedded in OCT compound, frozen, and stored at -70°C within 10 minutes of collection, until appropriate cryostat sections were cut.

CHAPTER III

METHODS AND TECHNICAL DEVELOPMENTS

A. JEJUNAL FLUIDS

Immunoglobulin and antibody assays

In specimens of jejunal fluid, concentrations of IgA and IgM, and levels of IgA and IgM antibodies to gliadin (GLI) (gift from Dr H. Weiser), ovalbumin (OVA) and beta-lactoglobulin (BLG) (Sigma Chemical, Poole, Dorset, UK) were assayed by enzyme-linked immunosorbent assays. To standardize the ELISA method for immunoglobulin total concentration, 20 samples (from the preliminary study) were used to assess the optical density coefficient of variation intra- and inter-assay, which ranged between 5-15%.

For assays of immunoglobulins in jejunal fluid specimens, serial 2-fold dilutions of a reference preparation were used to produce a standard curve, and serial dilutions of test samples were also assayed. Plates were read in an

MR5000 Automatic microplate reader (Dynatech, Billingshurst, Sussex, UK) set at a wave length of 405nm (OD^{405}). Only when the results of at least two of the sample dilutions fell within the range of the standard curve was the assay considered technically satisfactory, and the immunoglobulin content of the sample determined as the mean for these two sample dilutions. Standards used were: for IgA, human colostral IgA (Sigma Chemical Co. Poole, Dorset, UK); for IgM, human reference serum (Protein Reference Unit, Sheffield, UK).

In the assays of **specific antibodies**, prior experiments were carried out to determine optimum test conditions for each antigen, isotype and fluid. Serum from an untreated coeliac patient, previously recognized as having high levels of antibodies of IgG, IgA and IgM isotypes to GLI, OVA and BLG, was used as a reference standard. The reference specimen and test specimens were studied in duplicate at a predetermined dilution, varying for the different assays, and the plates read when the OD for the standard reached 1.0. If the duplicates varied by more than 10% the result was discarded. Antibody levels were expressed as percentages of the OD of the standard. Results are thus obtained and presented as non-parametric data; antibody levels are not directly proportional to the antigen-binding capacity of the sample. This is a feature of all such assays.

Assay for secretory IgA

Total and secretory IgA were quantified on the same microtiter plate by adding duplicate standards and fluid samples to plates coated with anti-human α chain (Sigma Chemical Co, Poole, Dorset, UK) and two secondary antibodies were used, an anti-IgA and a highly purified anti-secretory piece (The Binding Site, Birmingham, UK), both alkaline phosphatase AP-conjugated. Results were reported as total IgA concentration and the percentage of total IgA-bound to secretory component. To validate the test, and considering the possibility that unbound SC, or SC bound to secretory IgM, might compete for the binding sites of the ELISA-solid phase; it was made sure that SC which was unbound, or bound to IgM, was washed thoroughly away after the first incubation.

ELISA assay for IgM antibodies

Care was taken to ensure that the results were not merely due to non-specific binding of IgM, a matter of particular concern in assays of anti-GLI. All plates were pre-incubated with 1% bovine serum albumin in buffered saline with 0.5% Tween 20, pH 7.4, before addition of the samples. In general, the specificity of antibodies was tested by incubating samples with the appropriate protein

antigen and showing that the antibody was specifically absorbed out. To confirm antigen-specificity of the IgM assay, samples of jejunal fluid were tested for IgM anti-GLI and IgM anti-OVA, before and after incubation for one hour in gliadin-coated microtiter wells; there was 50-70% reduction of the optical density (OD) reading for anti-GLI but no change in anti-OVA; similar, antigen-specific reduction in activity was found after incubation in OVA-coated wells and testing for IgM anti-OVA and IgM anti-GLI antibodies (Figure 1).

Classification of antibody data

"Normal" values for the various antibody assays were based on results in 28 immunologically normal individuals previously studied (O'Mahony 1991a); as explained above, units of measurement were the OD for test specimen expressed as a percentage of the OD of the standard. For serum, normal values were taken as ≤ 20 for IgA anti-GLI, and ≤ 40 for IgG anti-GLI; for all IgA and IgM antibodies in jejunal fluid values ≤ 15 , were considered normal.

When jejunal fluid antibody studies showed a high value for IgM anti-GLI, together with high levels of at least two other intestinal antibodies characteristic of coeliac disease, ie. IgA anti-GLI, IgM anti-OVA, IgM anti-BLG, the specimen was designated as "coeliac-like intestinal

antibody" (CIA) positive, and all other specimens were considered CIA negative.

Total protein, albumin and alpha-1 anti-trypsin

Total protein concentration was assessed by a spectrophotometric method, the BCA (Bicinchoninic acid) Protein Assay (Pierce UK Ltd., Cambridge, UK), with protein standards provided by the kit, in jejunal fluid samples, diluted in bovine serum albumin. The standard protocol instructions were followed, a main incubation set up at 37⁰ C for 30 minutes, and microtiter plates were read at 562 nm.

Albumin was assessed by an immunoturbidimetric method, using an anti-human albumin antibody (Scottish Antibody Production Unit, Carlisle, Scotland). A similar method was used for α -1 anti-trypsin (α -1 AT), with an anti-human α -1 AT antibody obtained from the Protein Reference Unit (Royal Hallamshire Hospital, Sheffield, UK). Absorbency readings were set up at 340 nm for both assays.

TNF α and soluble IL-2R levels

I have used two commercial kits, the **TNF α -EASIA** (Medgenix Diagnostic, Brussels, Belgium), and the **Cellfree IL-2R ELISA** (T Cell Sciences, Cambridge, MA, USA). The TNF α -

EASIA is a solid phase "Enzyme Amplified Sensitivity Immuno-assay", which includes several mAbs against different epitopes of the TNF α molecule to increase sensitivity in a short incubation period. Standard control or jejunal fluids were added to microtiter plates coated with anti-TNF α . Following incubation at room temperature, anti-TNF α -HRP conjugate was added. After further incubation, the chromogen was added and the plates were read within one hour. Concentrations are extrapolated from the standard curve.

The Cellfree IL-2R ELISA kit is a sandwich enzyme immunoassay that includes two anti-IL-2R murine monoclonal antibodies. Standard control and jejunal fluids were added to microtiter plates coated with an anti-IL-2R mAb. After a second incubation with an anti-IL-2R mAb enzyme-HRP conjugate and addition of a chromogen, the ELISA-plate was read within an hour at 490 nm. Concentrations are extrapolated from the standard curve.

In the IFN γ -ELISA (developed by Louise Handy), plates were coated overnight at 4⁰ C, with anti-IFN γ mAb (Chromogenix AB, Molndal, Sweden), at dilution 1/50. Other incubations were done at room temperature. Duplicate standards and samples were added, and incubated for 3 hours. The 1st British Standard for human leukocyte

IFN γ (NBSB, NIBSC, Potters Bar, UK) was double diluted from 20-40 IU/ml. After overnight incubation with a biotinylated mAb to IFN γ (Chromogenix) at concentration 0.36 ug/ml; an ExtrAvidin Phosphatase conjugate (Sigma), at dilution 1/2000, was added for 1 hour. The reaction was visualized by adding 1 mg/ml of pnitrophenylphosphate substrate (Sigma) in 10% buffer diethanolamine, with plates shaking until standards OD reached 1.0 at 405 nm. The test was sensitive for levels >4 UI/ml of sample.

Sugar permeability test

A sample of urine was also collected from some patients, to perform a lactulose/rhamnose (L/R) jejunal permeability test (MacDonald 1991). Concentrations of lactulose and rhamnose in urine were assayed by High Pressure Liquid Chromatography and the percentage of lactulose and rhamnose excreted was expressed as the L/R ratio. Normal values are ≤ 0.040 .

B. JEJUNAL BIOPSIES

Counts of Intra-Epithelial Lymphocytes

A count was performed of lymphocytes within the villus or surface epithelium in H & E sections with a Leitz microscope and X100 immersion lens. At least 1000 enterocytes per biopsy were counted and the results were expressed as numbers of lymphocytes per 100 villus enterocytes, and normal values were considered to be between 10-40 IEL per 100 villus enterocytes (Ferguson & Murray 1971).

Counts of CD3 positive and of TCR $\gamma\delta$ positive IEL (series C,D)

Serial 7 μ m cryostat sections mounted on poly-l-lysine (Sigma, Poole, Dorset) coated slides, were dried, and then fixed in fresh acetone for 30 minutes at room temperature. After being rehydrated in Tris buffered saline (TBS, pH 7.6) and blocked with rabbit serum (Scottish antibody production Unit, SAPU, Carlisle, Scotland) in TBS, monoclonal antibodies were then applied for 60 minutes: TCR- δ 1 (T Cell Sciences, Cambridge, MA) at dilution 1/80, or CD3 (SAPU) at dilution 1/20;

followed by a Biotinylated rabbit anti-mouse IgG (DAKO, High Wycombe, UK) at dilution 1/400, for 60 minutes. The sections were treated with StrepABComplex (DAKO) for 60 minutes, and the reaction was visualized using diaminobenzidine as a substrate. Sections were counterstained with Gills number 1 haematoxylin. Optimal dilutions were selected following pilot studies using different antibody dilutions.

A Leitz-TAS plus computerized image analysis system, with a TASIC software operating system, was used for cell counts in frozen sections, and the results expressed as total cell count per mm of villous epithelium. Only those specimens in which at least 5 mm of epithelium could be counted were considered technically acceptable. Details of reference range derivation are presented below.

Counts of lamina propria plasma cells (series A)

An immunoperoxidase technique was used to stain immunoglobulin-containing cells. Briefly, sections were pre-treated with trypsin 0.1% in Tris buffered saline (pH 7.6) at 37⁰ C for 20 minutes, and then serially incubated with a primary antibody, sheep anti-human IgA or IgM (at 1/250 and 1/200 dilutions, respectively); a secondary antibody, affinity purified donkey anti-sheep/goat IgG (at dilution 1/500); and a tertiary antibody, sheep

peroxidase/anti-peroxidase (at dilution 1/80). All incubations were carried out in a humid chamber at room temperature for 60 minutes, and all antisera were obtained from the Scottish Antibody Production Unit (SAPU), Carlisle, Scotland. Positive and negative controls were included in each batch. Toluidine blue staining was used for mucosal mast cells.

Cell counts were performed blinded using a Leitz microscope with appropriate eyepieces and graticules, calibrated with a stage micrometer. Only well orientated sections were used, and fields were examined systematically, starting with the base of the graticule at the muscularis mucosae, counting sequential fields of lamina propria vertically to the luminal surface, then realigning the graticule on the muscularis mucosae and continuing the process. Epithelium was excluded, and where only part of a field comprised lamina propria, the proportion was estimated by eye. At least 40 lamina propria graticule fields were counted. The grid area with a x100 objective was 0.0132 square mm. The results were expressed as numbers of positive cells per square mm of lamina propria tissue (Arranz 1992).

Detection of HLA-DR, HLA-DQ and CD25 antigens in frozen

sections

According to previous reports, cell activation in gut mucosa may be manifest by HLA-DR expression by crypt epithelial cells; and by high numbers of CD25+ T cells and macrophage-like cells in lamina propria (Arnaud-Battandier 1986, Scott 1987, MacDonald & Spencer 1988, Haltensen & Brandtzaeg 1993). The intensity and distribution of HLA-DR+ cells in jejunal epithelium was assessed in relationship to the finding of a high total IEL count.

The immunohistochemical method is a three-stage streptavidin/biotin alkaline phosphatase (AP) technique with monoclonal antibodies (mAbs) to specific epitopes of the HLA-DR, HLA-DQ, and CD25 molecules (Immunotech, Marseille's, France). Sections were incubated with optimal dilutions of primary mAbs (1/100) for 40 minutes, then with a secondary antibody Biotinylated rabbit anti-mouse immunoglobulins Fab2 (DAKO, High Wycombe, UK) at dilution 1/300; and finally, treated with an streptavidin/biotin complex AP (StrepABComplex DAKO) for 60 minutes. The reaction was visualized using Fast Red TR AP-substrate (Sigma Chemical Co, Poole, Dorset, UK) containing levamisole 1M (Sigma) to block endogenous alkaline phosphatase, and N-N dimethylformamide. Sections were counterstained with Gills number 1 haematoxylin.

Sections were read blind on at least two different occasions within two months, using a Leitz microscope and appropriate objective lens, for a subjective evaluation of the intensity, number and distribution of positive cells. For every biopsy, sequential cryostat sections stained for CD3, HLA-DR, HLA-DQ, and CD25, were evaluated, as well as two control sections, negative (sequential section stained with the same protocol, except the specific antibody) and positive (section from an untreated coeliac patient). Staining results were scored as negative (0), positive weak (1), strong (2), very strong (3); and an estimate of the number of positive cells was recorded by different areas of distribution: villus epithelium (VE), villus lamina propria (VLP), crypt epithelium (CE), and pericryptal area (CA).

Disaccharidase levels

If the biopsy specimen was large enough, a separate piece of tissue was taken for assays of disaccharidases: lactase, sucrase, maltase and trehalase (Dahlquist 1968).

C. STATISTICAL METHODS

The Student t test and the Mann-Whitney U test were used respectively for comparing immunoglobulin and antibody levels between groups; and the Spearman's test for correlations between serum and jejunal fluid anti-GLI antibody levels. P values of <0.05 were considered significant. Differences in cell counts between groups in **series C,D**, were assessed by the two-tailed Mann-Whitney U test. Comparisons of frequency tables of positivity for candidate markers using the Chi square test with Yates' correction.

SECTION THREE

RESULTS

CHAPTER IV

STUDIES IN JEJUNAL FLUIDS

Introduction

Jejunal fluid is an homogeneous secretion obtained under controlled conditions from a point distal to the duodenal-jejunal junction. Basically, intestinal fluid is drained by gravity through the tubing of the Watson biopsy capsule, and collected during a fixed period of time (5 minutes) into a recipient tube which contains 20 ul/ml of the protease inhibitor phenylmethyl sulphonyl fluoride (PMSF) at a concentration of 0.1 mol/l, to preserve the protein content. Jejunal fluid is transferred on ice to the laboratory and stored at - 70⁰ C within 10 minutes.

Many studies have been published on immunoglobulin and antibody measurements in jejunal fluid aspirates, but they are uncritical in assuming the validity of this fluid as a local secretion, and also in the application of immuno-assays (ELISA) and other tests previously used in serum to jejunal fluid without adapting them to the

special characteristics of this secretion. This chapter brings together several technical and validation experiments carried out on our samples.

Detection of sugar probes in jejunal fluids

Jejunal fluids are collected at the time of performing the sugar permeability test (using two sugar probes, lactulose and rhamnose). The question arose of whether this procedure was technically correct, because the probes might also be aspirated with the fluid. The results, obtained from a group of 10 samples, suggest that only 2-5% of the calculated amount given is found in jejunal fluid aspirate. It is important to point out that our normal values are based on individuals in whom a sugar test is performed at the same time as jejunal fluid collection. Therefore, if further work in other conditions is to be carried out, normal ranges in jejunal secretions would have to be re-calculated.

Jejunal fluids taken through the tubing of a biopsy capsule may be different from those obtained in the endoscopy theatre (fasting state). It is possible that the latter are less diluted, and therefore they would have a higher concentration of immunoglobulins or antibodies. However, this will have to be investigated by comparing samples from endoscopy and those taken by our

routine procedure in the course of a diagnostic jejunal biopsy.

Determination of plasma protein leakage: total protein, and albumin and α -1 anti-trypsin levels

For a proper interpretation of the studies in jejunal fluid, it was necessary to assess how much of the jejunal fluid volume obtained is contributed by plasma leakage, and whether this was different in the various diagnostic groups, such as coeliac disease or inflammatory bowel disease (IBD). Others in our group (O'Mahony 1991b, Brydon 1993) have developed assays for plasma derived proteins, albumin and alpha-1 anti-trypsin, in whole gut lavage fluid, and they were ready to be applied to jejunal fluid specimens. Whole gut lavage fluid is a gut perfusate with a rate of 20 ml/per minute. Normal ranges for albumin (<1-26 ug/ml) and α -1 AT (<1-19 ug/ml) have been described for gut lavage samples obtained from a group of individuals with minor GI symptoms or considered to be immunologically normal (Brydon 1993).

Intestinal secretions may contain IgG related to certain diseases, and IgG seems to be a specific index of disease activity in gut lavage fluid from IBD patients (O'Mahony 1991b, Choudari 1993). The IgG is probably derived from plasma because of the correlation found between IgG and

albumin levels. Faecal α -1 AT concentration and clearance have been previously used as indices of gastrointestinal protein loss.

- Description of cases, results and discussion

Total protein content, expressed as mg per ml of sample, was tested in jejunal fluid specimens from 14 patients (10 women, 4 men), 3 of them were treated coeliacs and one was an untreated coeliac, one patient has DH, and 9 cases had other disorders (description of cases and results are shown in Table IV.1). Albumin and α -1 AT levels were tested in 36 patients (27 women, 9 men), of whom 11 were coeliacs (5 untreated and 6 treated); 15 were non-coeliacs patients with a variety of GI diseases such as IBD, idiopathic diarrhoea, or mouth ulcers; and 10 were patients who had a functional bowel disorder, or in whom no diagnosis was reached after full investigation (description of cases are shown in Table IV.2).

Reference ranges for jejunal fluid specimens were set up using the group of 10 non-coeliac patients with minor GI symptoms considered to also be immunologically normal. This range was calculated using the 95% confidence interval: 1-65 ug/ml for albumin and 1-21 ug/ml for α -1 AT. Results are shown in Table IV.3. Untreated coeliac patients had higher albumin levels when compared to non-

TABLE IV.1. DESCRIPTION OF CASES AND RESULTS OF TOTAL PROTEIN CONCENTRATIONS IN JEJUNAL FLUID.

DIAGNOSIS	AGE	DIET	BIOPSY HISTOLOGY	PROTEIN (mg/ml)
Coeliac disease	62	GFD	PVA	3.4
Coeliac disease	33	GFD	PVA	5.3
Coeliac disease	37	GFD	Normal	1.2
Coeliac disease	37	N	TVA	3.5
Dermat.herpetiformis	52	N	Normal	1.1
Ulcerative colitis	20	N	Normal	3.5
Idiopathic diarrhoea	44	N	Normal	2.4
Idiopathic diarrhoea	21	N	Normal	0.4
Excess alcohol	54	N	Normal	1.9
Diverticulitis	78	N	Normal	3.3
Travellers' diarrhoea	30	N	Normal	3.4
Coagulation disorder	44	N	Normal	4.5
Anaemia (Fe def)	41	N	Normal	4.5
IBS	25	N	Normal	5.2

TABLE IV.2. DEMOGRAPHIC DATA OF PATIENTS STUDIED FOR ALBUMIN AND α -1 ANTI-TRYPSIN IN JEJUNAL FLUID.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD	HISTOLOGY NORMAL MINOR PTVA CHANGES
COELIAC Untreated	5	49 (37-57)	3/2	5	5
Treated	6	45 (33-62)	3/3	6	4 2
IBD	4	43 (20-75)	3/1	4	4
DIA IDIO	6	46.5 (31-66)	5/1	6	6
ORAL ULC	1	23	1/-	1	1
MISC	3	30 (26-33)	3/-	3	2 1
NUT DEF	2	40 (25-55)	2/-	2	2
IBS	7	35.6 (20-59)	5/2	7	7
NAD	2	44.5 (21-55)	2/-	2	2

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

TABLE IV.3. ALBUMIN AND α -1 ANTI-TRYPSIN LEVELS IN JEJUNAL FLUID

	ALBUMIN (ug/ml)	α -1 ANTI-TRYPSIN (ug/ml)
COELIAC PATIENTS		
Normal diet (n=5)	*161.4, 105 (52-300)	8.6, 6.4 (1-18)
GFD (n=6)	40.5, 57.3 (4-156)	5.2, 4.6 (0-10)
CONTROL GROUP (n=10)	26.3, 19.7 (9-93)	7.8, 6.4 (0-22)
ORGANIC DISEASE GROUP (n=14)	32.2, 30 (0-93)	15.5, 18 (1-67)

* Coeliac patients had higher albumin concentrations ($p < 0.001$) than control and organic disease groups.

Data expressed by mean, standard deviation (range values)

coeliac patients with either an organic GI disease or controls (both, $p < 0.01$) This "normal" range for jejunal fluid albumin is twice the value reported for whole gut lavage samples, whereas α -1 AT levels are similar in both fluids. Protein concentration in jejunal fluid samples studied ranged from 0.4 to 5.3 mg/ml (mean value 3.1).

Eleven of the 36 patients studied had albumin levels above the upper range limit, these include 6 coeliac patients (all 5 untreated, and one treated patient with PVA). In coeliac disease, the results may be explained by the existence of an increased intestinal permeability. Five non-coeliac patients had high albumin levels (a description of the non-coeliac cases is shown in Table 12), 4 were CIA positive: 2 diagnosed as having idiopathic diarrhoea and an abnormal permeability test; one of gastritis and a high IEL count; one of recurrent mouth ulcers; the fifth CIA negative case had ulcerative colitis. Alpha-1 anti-trypsin levels were high in 4 non-coeliac patients (Table 12): one patient had idiopathic diarrhoea, a high level of jejunal fluid albumin, and an abnormal permeability test; there were 2 IBD patients (one had high albumin levels); and a woman with minor symptoms who was convinced that she had multiple food allergies, though we had no evidence to support this.

TABLE IV.4. DESCRIPTION OF NON-COELIAC CASES WITH HIGH LEVELS OF ALBUMIN AND α -1 ANTI-TRYPSIN IN JEJUNAL FLUID

DIAGNOSIS	AGE	BIOPSY HISTOLOGY	CIA	PERMEABILITY TEST	ALBUMIN	α -1 AT
Idio Dia	51	Normal	+	+	+	+
Idio Dia	82	Normal	+	+	+	-
Oral Ulc	47	Normal	+	-	+	-
UC	75	Normal	-	-	+	+
CRO	50	Normal	+	-	-	+
Delusion	32	Normal	-	-	-	+
Nad	26	High IEL	+	-	+	-

Measurement of Secretory IgA

The measurement of secretory IgA in jejunal fluids, as the percentage of total IgA bound to secretory component (SC), may serve as an indirect indication of local immunoglobulin secretion, given that non secretory component bound IgA, might be derived from serum leakage. Secretory component is a transmembrane glycoprotein receptor for polymeric immunoglobulins, synthesized and expressed by intestinal epithelial cells (Brandtzaeg 1974; Brown 1976, 1977), that can also be found in secretions as a free molecule or bound to polymeric immunoglobulins (IgA and IgM) (Delacroix & Vaerman 1981).

Secretory IgA, IgM, and SC are antigenically different for the specificities of the polyclonal antibodies used in immunoassays. Human SC epitopes are differently expressed in sIgA, being more inaccessible because of the covalent properties needed for binding and stabilization of the molecule (Solari 1985). The presence of free SC may be a problem in the measurement of secretory immunoglobulins by immunoassay, because it might block binding to the solid phase. Circulating free SC is almost absent from serum (perhaps only detected in cases of hypogamma-globulinaemia), and the different binding properties of SC versus SC-IgA-bound, reduce the

possibility of competition for the coated antisera in serum estimations.

Free SC may also be a problem in the quantification of sIg in fluids by immunoassay, because this molecule may compete with sIgA for the binding sites on the solid phase, and therefore it may give a reduced estimation of the actual sIgA concentration. When secretions are tested, the inhibitory effects between SC-bound pIgs may be overcome by coating the microtiter plates with an excess of anti-SC antibody, and reducing the level of sIgM and sIgA by appropriate dilution; or by using an anti- α chain as a coating antibody (Chapter VI). Low free-SC levels have been reported in most biological fluids, such as sera, urine, whole saliva, and jejunal fluid using specific coating mAbs (Vincent & Revillard 1988).

Secretory IgM can be removed from intestinal secretion samples by absorption using immunosorbent columns containing specific anti-IgM antibodies (Bartholomeusz 1989). An alternative method coats microtiter plates with specific anti- α chain antibody, or different mAbs specific for sIgA and sIgM, then after binding uses an anti-SC conjugate as a secondary antibody (Ishiguro 1981, Hjelt 1988).

- Description of cases, results and discussion

A group of unselected jejunal fluid samples from 20 patients were tested for sIgA as a percentage of the total IgA concentration. These included 12 women and 8 men, with an age range of 15-67 (mean: 33.7); of whom 3 were coeliac or DH patients, 12 had other GI diseases (IBD, idiopathic diarrhoea, nutritional deficiencies, others), and in 5 cases no abnormality was detected after further investigation. Description of cases and results are shown in Table IV.5. Secretory IgA levels ranged from 58 to 98% of the total IgA, with a mean value of 74.7% (SD 11.2). No significant differences were found when CIA positive (8 cases, mean value of sIgA: 75.4%), and CIA negative patients (12 cases, mean value of sIgA: 72.6), were compared.

Previous results using the same specific ELISA in a population of elderly and control patients (Arranz 1992) showed that almost all salivary IgA was bound to the SC (99% of the total IgA concentration), the percentage decreased to 70-90% in jejunal fluid, and it was even lower in whole gut lavage samples (65%). Since leak of plasma-derived proteins, such as albumin and α -1 anti-trypsin, was unusual, this 10-30% of unbound IgA detected in jejunal fluid cannot be 7S-IgA (monomeric) derived from plasma. However, it might be 10S

TABLE IV.5. DESCRIPTION OF CASES AND RESULTS OF JEJUNAL FLUID TOTAL IgA AND SECRETORY IgA AS PERCENTAGE OF THE TOTAL

DIAGNOSIS	AGE	CIA	BIOPSY HISTOLOGY	IMMUNOGLOBULIN A TOTAL (ug/ml)	SECR (%total)
Coeliac (N diet)	28	+	STVA	663	76
DH (N diet)	43	+	Min chan	926	90
DH (N diet)	61	+	Min chan	481	82
UC.Scler.cholangitis	24	+	Min chan	155	97
UC	28	-	Normal	466	82
Idio. diarrhoea	15	-	Normal	277	55
Idio. diarrhoea	19	+	Min chan	624	74
Idio. diarrhoea	28	+	Normal	469	58
Idio. diarrhoea	32	+	Min chan	911	63
Gastric surgery	26	-	Normal	176	83
Pancreatitis	61	-	Normal	96	75
Rheum. arthritis	39	-	Normal	212	73
Nutr. deficiency	44	+	Min chan	289	83
Fe def. anaemia	23	+	Normal	913	80
Fe def. anaemia	67	-	Normal	192	74
IBS	29	+	Normal	581	71
Nad	32	-	Normal	845	68
Nad	38	-	Normal	118	55
Nad	19	+	Min chan	233	80
Nad	18	-	Normal	230	73

Min Chan= high total IEL counts

monomers) or 11S (dimeric) IgA.

The results of antibody levels in jejunal fluids (see Chapters VIII and IX) were broadly similar to those in other intestinal secretions, eg whole gut lavage fluid; and a positive correlation was found for IgA anti-gliadin antibody levels in patients studied by both techniques (O'Mahony 1991a). Immunoglobulin concentrations, however, differed markedly in jejunal and gut lavage fluids, whereas total IgA, IgM and IgG were elevated in jejunal fluid from untreated coeliacs. Only IgM was significantly elevated in gut lavage fluid. In this respect, whole gut lavage samples are a less homogeneous fluid (it is likely to contain gastric juice, bile, and secretions from pancreas, small bowel, and colon), as compared to jejunal fluid, which may reflect more accurately local immune phenomena in the jejunum only.

Antigen-related terminal differentiation and expansion of IgM and IgA B cells appear to be normal in non-coeliac CIA positive patients. Trans-epithelial immunoglobulin transport is also normal (Baklien 1977), indeed the production of SC by epithelial cells is increased in coeliac disease (Scott 1981). An abnormally high capacity for transport of IgM and IgA across the epithelium might produce the CIA pattern. However, this is not supported by the finding of a similar proportion of jejunal fluid

IgA bound to SC in CIA positive and CIA negative samples. The pentameric IgM molecule may have a stronger non-covalent interaction with SC than dimeric IgA (Brandtzaeg 1985, Bouvet 1990). Thus, over-production of IgM in the lamina propria may partially block the trans-epithelial IgA transport, an explanation for the presence of dimeric IgA in the serum in coeliac disease (Troncone 1991b).

CHAPTER V

HUMORAL SECRETORY IMMUNITY IN COELIAC DISEASE

Introduction

The initial part of this work, which involved the study of intestinal humoral immunity in coeliac disease, was done in collaboration with Dr Seamus O'Mahony, and we worked together in laboratory testing and evaluation of data. Previous studies in the literature had focused on systemic humoral immune responses, whereas information on local mucosal immunity was scarce. Circumstantial evidence of the existence of a local cell mediated response to gluten, had been reported (MacDonald & Ferguson 1975). Immunohistochemical studies showed that untreated coeliac patients had increased numbers of IgA and IgM jejunal plasma cells, but also IgG (Baklien 1977, Wood 1987); and coeliac children had antibodies to dietary antigens in intestinal secretions (LaBrooy 1986, Volta 1988).

The aim of the study was to characterize the secretory antibody responses in vivo, and establish type-specific

values for total immunoglobulins and antibodies to gliadin and other common dietary antigens in well characterized groups of treated and untreated coeliacs and non-coeliac controls. The results were evaluated to determine if the changes observed were intrinsic and due to a primary abnormality in coeliac intestinal immunity (eg found in treated and untreated patients) or, on the other hand, if the changes were secondary to the lesion (eg only in untreated patients). Eventually, systemic and mucosal immune responses (serum vs jejunal fluid samples) were compared to test the value of these local secretions in the study of GI immunity in coeliac disease and other disorders.

Immunoglobulin concentrations in sera and jejunal fluids

No significant differences were observed in serum samples when coeliac patients and controls were compared. However, untreated coeliacs had significantly higher jejunal fluid concentrations of IgA, IgM and IgG when compared to controls, and higher IgM when compared to treated coeliacs (Table V.1). Values for treated coeliacs were not significantly different from controls. Of interest is the fact that the IgM content in gut lavage fluid was also found to be significantly higher than controls in both untreated and treated coeliacs (O'Mahony 1991a).

TABLE V.1. IMMUNOGLOBULIN CONCENTRATIONS IN SERUM AND JEJUNAL FLUID

		UNTREATED COELIACS (n=26)	TREATED COELIACS (n=15)	CONTROLS (n=28)
SERUM (mg/ml)	IgA	2.4 (1.3-3.8)	2.0 (0.7-3.3)	1.8 (0.9-4.1)
	IgM	1.1 (0.3-3.4)	0.9 (0.3-3.1)	1.3 (0.3-4.5)
	IgG	10.5 (5.6-15.7)	10.7 (5.4-20.1)	9.5 (7.3-15.9)
JEJUNAL FLUID (ug/ml)	IgA	*290 (25-2285)	140.7 (20-1558)	102.5 (23-540.6)
	IgM	*+29.8 (1.9-358)	9.6 (0-174)	4.5 (0-39.5)
	IgG	*23.9 (3-261.8)	11.8 (0.3-271.4)	7.3 (1.2-29.3)

*Immunoglobulin concentration higher (p<0.05) than in controls
+Immunoglobulin concentration higher (p<0.05) than in treated coeliacs
Data expressed by median and range values

Food antibody levels in serum and jejunal fluid samples

High levels of serum IgA anti-gliadin antibody were found in untreated coeliacs, whereas treated coeliacs have similar values to controls. Both untreated and treated coeliacs had high levels of serum IgG anti-gliadin antibody, with significantly higher levels in the untreated group. There were no significant differences between patient groups in levels of serum IgM antibodies. Patterns of serum antibodies to BLG and OVA were generally similar to those for anti-gliadin antibody (Table V.2). Untreated coeliacs had high values for IgA anti-BLG and anti-OVA and IgG anti-BLG antibody. Serum IgA anti-BLG and IgG anti-OVA antibody levels were higher in treated coeliacs than controls.

In jejunal fluids, there was very little antibody detected in samples from controls, but as detailed in Table V.3, for all 3 isotypes, and all 3 antigens studied, levels of antibody were significantly higher in jejunal fluids from untreated coeliacs than in controls ($p < 0.02$ for all). For anti-gliadin antibodies, levels in treated coeliacs were intermediate between untreated coeliacs and controls and significantly different from both. When antibody levels in the 11 treated patients with entirely normal jejunal histology were compared with

TABLE V.2. SERUM FOOD ANTIBODY VALUES

		UNTREATED COELIACS (n=26)	TREATED COELIACS (n=22)	CONTROLS (n=28)
GLIADIN	IgA	*+43.7 (4.6-150)	5.8 (0.3-41.9)	5.3 (0-44.5)
	IgM	43 (19.3-95.4)	38.6 (10.5-87.6)	47.9 (16.1-109)
	IgG	*+82.4 (9.8-137.4)	*41.6 (6.8-107.4)	21 (0-105.2)
BLG	IgA	*+14.7 (1.4-150)	*6.6 (2.1-87.5)	3.3 (0.2-36.4)
	IgM	33.6 (9-125.5)	38.7 (11.3-94.6)	27.4 (3.5-82.2)
	IgG	*94.7 (5-150)	70.2 (8.6-150)	30.9 (0-131.8)
OVA	IgA	*18.8 (2.6-150)	14.8 (4.8-60.6)	11.4 (0.9-35.6)
	IgM	31.1 (4-87)	37.8 (9.5-123.4)	30 (5.70-102.6)
	IgG	64.4 (2.6-150)	*63.4 (8.4-150)	27.6 (3.6-150)

*Antibody values higher ($p < 0.05$) than in controls

+Antibody values higher ($p < 0.05$) than in treated coeliacs

BLG=betalactoglobulin

OVA=ovalbumin

Data expressed by median and range values

TABLE V.3. JEJUNAL FLUID FOOD ANTIBODY VALUES

		UNTREATED COELIACS (n=26)	TREATED COELIACS (n=22)	CONTROLS (n=28)
GLIADIN	IgA	*+26.5 (7.7-134.5)	*4 (0.3-34.2)	1.1 (0-13.3)
	IgM	*+80.5 (0.1-150)	*19.6 (2.1-114.4)	1.9 (0-11.7)
	IgG	*+1.8 (0-23.4)	*1 (0-4.8)	0.2 (0-2.2)
BLG	IgA	*25.3 (0.6-150)	9.2 (1.1-110.9)	5 (0.1-60.8)
	IgM	*16.4 (0-150)	*9.2 (1.1-78.3)	1.6 (0-11.7)
	IgG	*4.5 (0-104)	1.6 (0-35.1)	1 (0-7.7)
OVA	IgA	*15.8 (4-150)	*19.6 (1.5-66.5)	4.2 (0-100.5)
	IgM	*18.7 (0-97)	*8.4 (0.1-52.5)	1.1 (0-17.7)
	IgG	*1.3 (0-26.2)	1.1 (0-11.7)	0.5 (0-26.5)

*Antibody values higher ($p < 0.05$) than in controls

+Antibody values higher ($p < 0.05$) than in treated coeliacs

BLG=betalactoglobulin

OVA=ovalbumin

Data expressed by median and range values

controls, IgA anti-gliadin antibody levels were not significantly higher. Conversely, levels of IgM anti-gliadin antibody remained significantly elevated ($p < 0.005$) in this group. Antibodies to BLG and OVA showed a greater overlap between levels in coeliacs and controls, but again high levels of IgM antibodies persisted in the treated patients. Jejunal fluid IgA and IgM anti-gliadin antibody levels are shown in Figure V.1.

Serial studies in coeliac patients

Seven coeliac patients who had a clinical and histological response to a GFD were studied before and after treatment. Serial changes in serum IgA anti-gliadin antibody and jejunal fluid IgA and IgM anti-gliadin antibodies are shown in Figure V.2. Whereas serum antibody levels fell significantly ($p < 0.05$) with treatment, there was no significant change in levels of intestinal antibody despite histological recovery on a GFD. It should be noted that these 7 patients had been taking a GFD for 6 months or less; this may account for the fact that they have different results from other treated coeliac patients on a GFD for a longer period, who had significantly lower jejunal fluid antibody levels compared to untreated coeliacs.

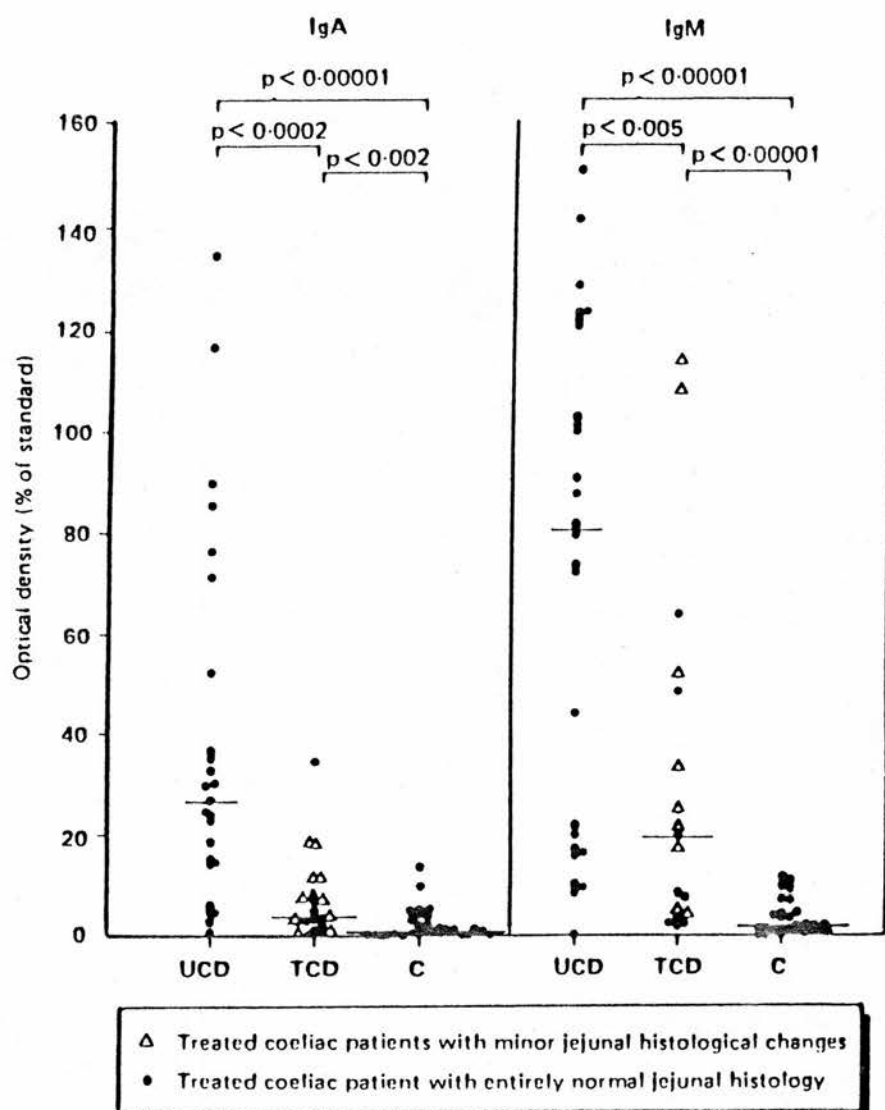


Figure V.1: Jejunal fluid IgA and IgM anti-gliadin antibody values (mean antibody values shown as horizontal bars).

UCD=untreated coeliacs, TCD=treated coeliacs, C=controls

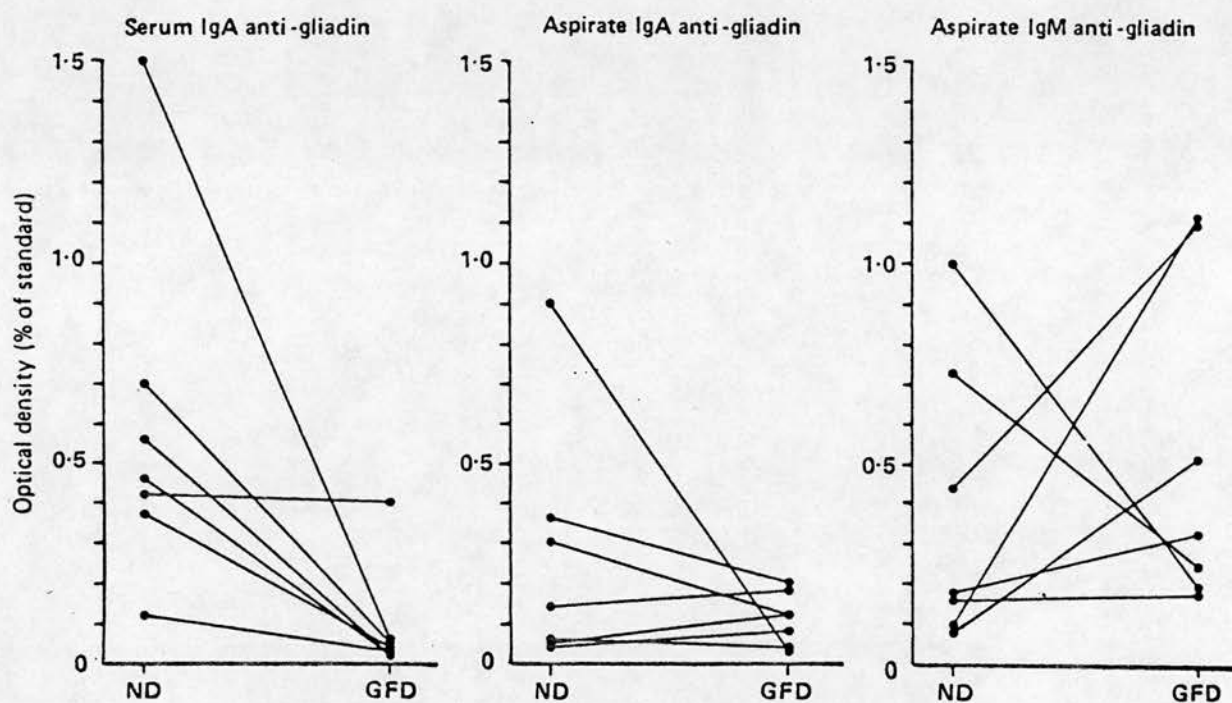


Figure V.2: Serial changes in values of serum anti-gliadin antibody and jejunal fluid IgA and IgM anti-gliadin antibodies in 7 coeliac disease patients before and after treatment with GFD. ND= normal diet; GFD= gluten-free diet.

Correlations between antibody levels in serum and jejunal fluids

IgA anti-gliadin antibody levels were compared in serum and jejunal fluid samples. In the untreated coeliacs, we found a positive correlation between antibody levels in serum and jejunal fluid ($r=0.68$, $p<0.0001$) but in the treated coeliacs, this correlation was not maintained, as treated coeliacs had high levels of jejunal fluid antibody, whereas serum antibody levels were low or absent.

Discussion and implications of these results

Intestinal anti-gliadin antibodies in coeliac patients are mainly of the IgA and IgM classes, and significant amounts of IgM antibody persisted in the secretions of treated coeliacs with entirely normal jejunal histology. Secreted IgA antibody was detected only in the subgroup of treated coeliacs with minor histological abnormalities, most of whom had been less than a year on treatment. It is possible that the intestinal IgA anti-gliadin antibody and minor histological changes could both be due to continued ingestion of small amounts of gluten. Conversely, intestinal IgM antibody levels remained higher than controls even in patients with

completely normal jejunal mucosa who had been taking a GFD for some years. It is likely that minute amounts of gluten maintain a local immune response rather than a systemic one. The finding of a persistent IgM antibody response parallels the finding of a relatively high fraction of IgM+ plasma cells in treated coeliacs (Baklien 1977; Scott 1984, 1992).

Concentrations of IgA, IgM and IgG were all high in jejunal fluids from untreated coeliacs. Counts of jejunal plasma cells and in-vitro immunoglobulin production in coeliac disease are higher than in controls for all isotypes (Lancaster-Smith 1976, Scott 1984, Wood 1987), supporting the view that the immunoglobulins (at least IgA and IgM) in coeliac intestinal secretions are produced locally. It is likely that most of the jejunal IgG is plasma-derived, as numbers of IgG-secreting plasma cells are low even in untreated coeliac disease (Baklien 1977). It should be pointed out that immunoglobulin measured in jejunal fluid is total immunoglobulin, and not specific secretory IgA and IgM. It is conceivable that at least some of the jejunal IgA and IgM is serum-derived (coeliac disease is a protein-losing disorder). Other studies have been performed to characterize jejunal immunoglobulins and antibodies in terms of molecular weight and percentage of total Ig which contains SC. Elevation of intestinal fluid immunoglobulin

content was not accompanied by equivalent changes in serum immunoglobulins; in fact, serum IgM content was markedly low in some untreated coeliacs.

In any event, enhanced intestinal antibody production in coeliac disease is not limited to gluten-derived proteins. High levels of intestinal antibody to BLG and OVA in untreated coeliacs were also found, with persistence of IgM antibody to these proteins in treated coeliacs. Antibodies to these food proteins were less specific to coeliac disease than anti-gliadin antibodies. It has been suggested that high levels of serum antibody to these proteins in untreated coeliacs are simply the result of increased intestinal permeability to antigens.

Although ELISA is now accepted as the standard assay technique for measuring antibodies to food proteins in coeliac disease, several different ELISA methods have been described, and different reference preparations are used by each group of investigators. The method employed is essentially similar to one previously reported (Savilahti 1983), and I used crude gliadin (instead of α -gliadin) as antigen for the ELISA, as others (Skerrit 1987) have shown that sera and intestinal fluid from coeliac patients contain antibodies which bind to several different gliadin subunits. Levels of positivity or negativity have not been ascribed to antibody levels;

such arbitrary designations are of some value in screening tests used in clinical practice but not in prospective research investigations.

It is notable that antibody findings in jejunal fluids were broadly similar to those in other intestinal secretions, eg gut lavage fluid; and a positive correlation was found for IgA anti-gliadin antibody levels in patients studied by both techniques (O'Mahony 1991a). Immunoglobulin concentrations, however, differed markedly in jejunal and gut lavage fluids, all 3 immunoglobulin isotypes were elevated in jejunal fluid in the untreated coeliacs, whereas only IgM was significantly elevated in gut lavage fluid. Gut lavage fluid is likely to contain secretions not only from the small bowel, but also gastric juice, bile, pancreatic secretions and colonic secretions. In this respect, it is not a homogeneous fluid, unlike jejunal fluid, which reflects events in the jejunum only, and is thus more likely to represent accurately local immune phenomena in coeliac disease. Fluid flow rate is another factor which may influence immunoglobulin levels in exocrine secretions; this may partly account for differences in findings in jejunal and whole gut lavage fluids.

The results of this preliminary study in coeliac patients lead to the definition of a "coeliac-like intestinal

antibody" (CIA) pattern, as the presence in jejunal fluid of IgM anti-gliadin together with at least two of the three other characteristic antibodies, IgA anti-GLI, IgM anti-OVA and IgM anti-BLG.

The study also demonstrates the virtual independence of systemic and intestinal humoral immune responses, and shows that for the study of immunopathology of intestinal disease, direct investigation of the gut is mandatory.

This intestinal antibody pattern persists during strict gluten exclusion in coeliac patients. The finding of a similar antibody pattern in DH patients without enteropathy (O'Mahony 1990a), suggests that non-DH latent coeliacs might also be CIA positive.

CHAPTER VI

HUMORAL SECRETORY IMMUNITY IN SYMPTOMATIC PATIENTS REFERRED AT THE G.I. UNIT

Introduction

It has been shown in the previous chapter that treated coeliacs with healed mucosa have a similar pattern of intestinal antibodies to untreated coeliacs. A similar pattern has been described in DH patients without enteropathy (O'Mahony 1990a). It was thought that this antibody pattern might recognize latent coeliac disease amongst non-coeliac patients. Between 200-250 patients per year are referred to this unit for diagnostic small bowel investigations, under the suspicion of coeliac disease. In order to facilitate research on latent coeliac disease, I organized the collection of specimens of serum and jejunal fluid at the same time as the jejunal biopsy was taken. Eventually, the majority of these patients are found to have a morphologically normal biopsy, or only an unexplained high IEL count.

I studied the expression of the coeliac-like intestinal

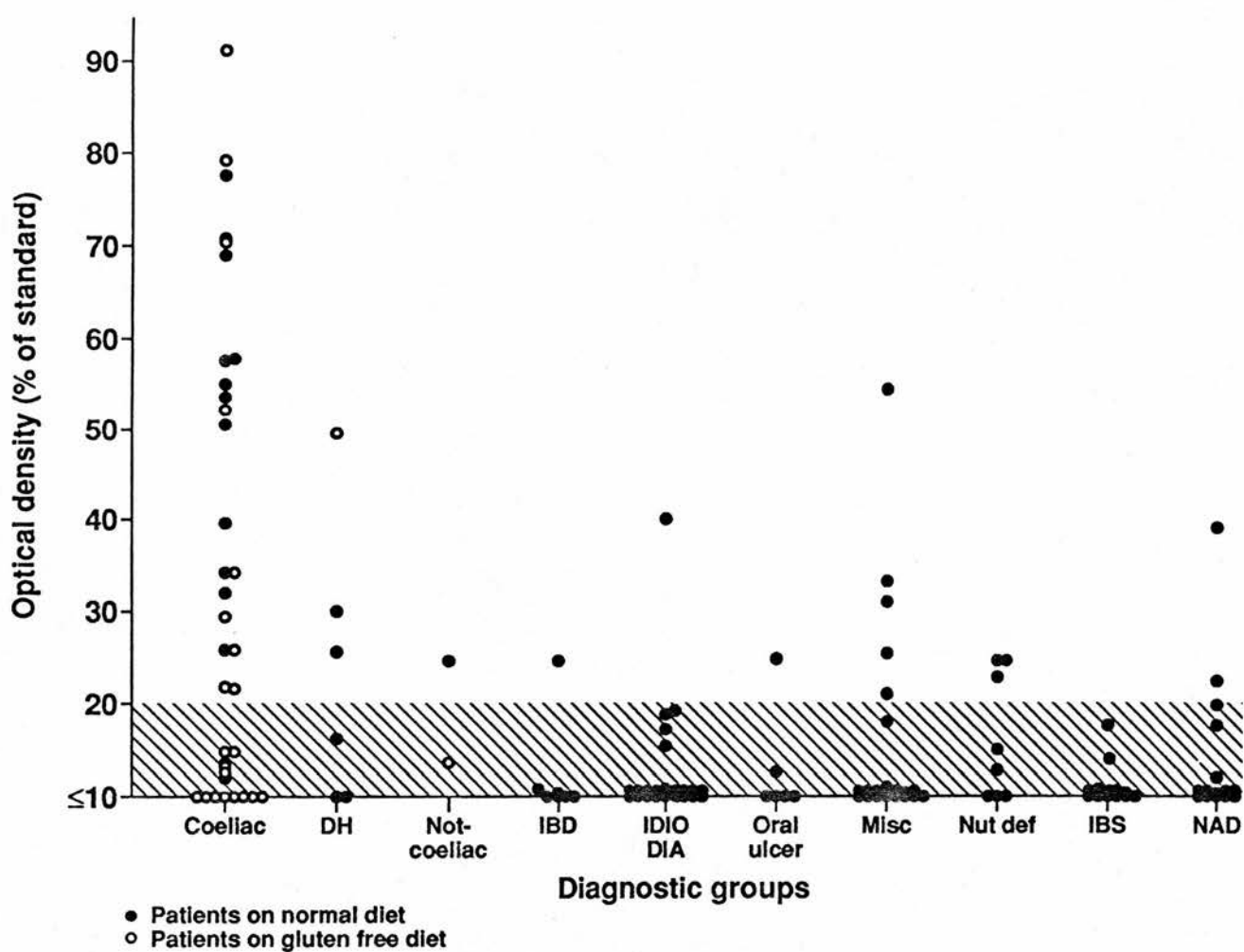
antibody - CIA - pattern in this prospective cohort of non-coeliac patients, to assess its frequency in this total population and also its relative expression by different non-coeliac diagnostic groups. Of potential interest was to find out if cases of gluten-sensitivity in which classical enteropathy was not present, might be found in this way. However, in this part of my work, I studied only the presence of the CIA pattern and how it related to serum anti-gliadin antibodies; studies of clinical gluten sensitivity were carried out separately.

Characterization of the CIA pattern

The "coeliac-like intestinal antibody" (CIA) pattern was defined as the presence in jejunal fluid of IgM anti-GLI together with at least two of the three other characteristic antibodies, IgA anti-GLI, IgM anti-OVA and IgM anti-BLG. This antibody pattern does not correlate with antibody levels in serum, when samples of jejunal fluid and serum are compared in the same patient.

Anti-gliadin antibody levels in sera, series A

Serum samples, collected at the time of jejunal biopsy, were available for 137 of the 140 cases studied. Levels of serum IgA and IgG anti-gliadin antibodies are shown in Figures VI.1 and 2. Twelve of the 14 untreated coeliacs



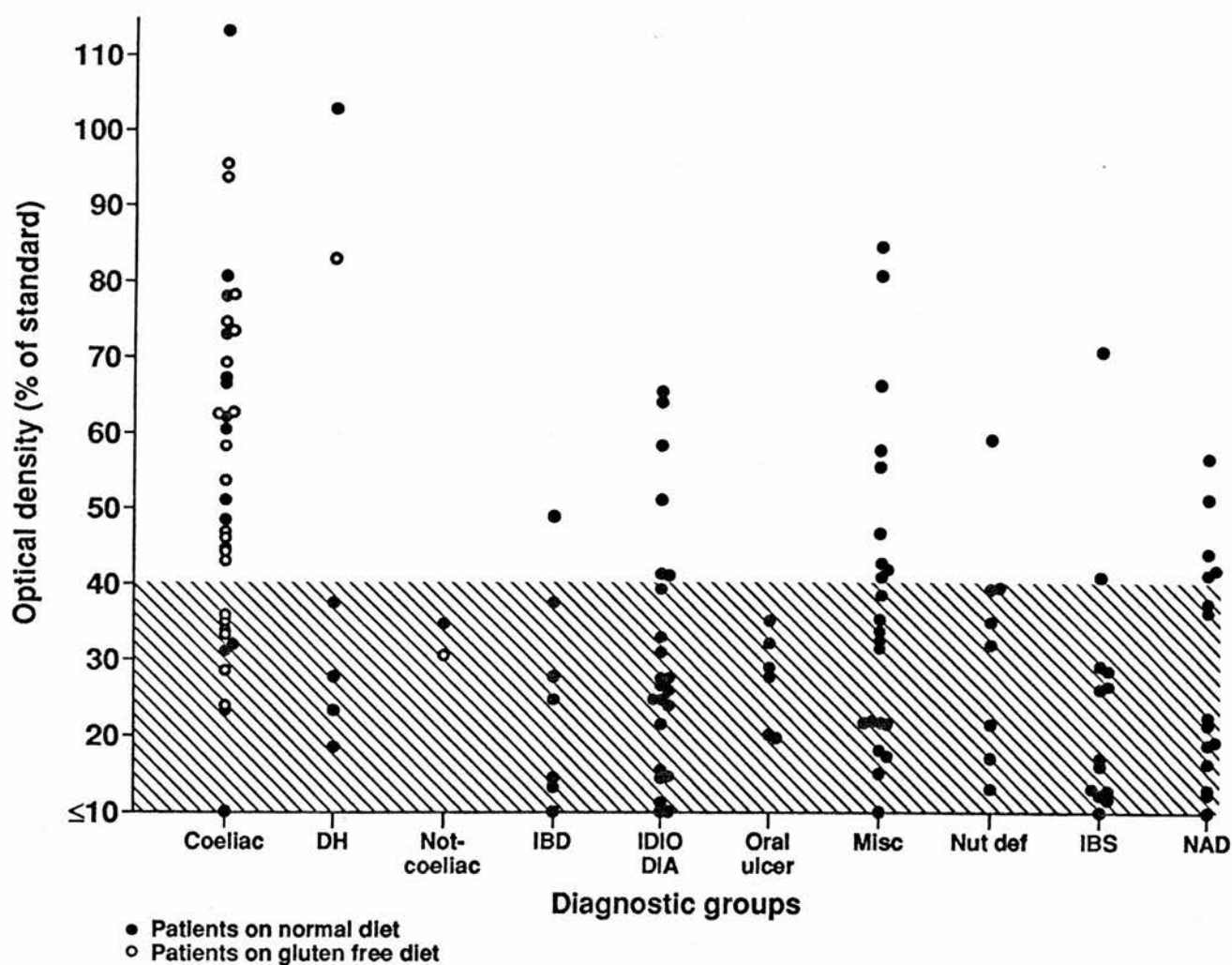


Figure VI.2: Serum IgG antigliadin antibody levels in patients grouped by final diagnosis (Normal range is shaded).

had high levels of serum IgA anti-gliadin, as did 9 of the 21 coeliacs on a GFD. Of the 5 new DH cases, two had high serum IgA anti-gliadin, and one had high serum IgG anti-gliadin. Fourteen of the 96 non-coeliac, non DH cases also had IgA anti-gliadin values above 0.20, but, as illustrated, in only one was the level particularly high, a man with alcoholic cirrhosis. In contrast, detectable serum IgG anti-gliadin was present in the majority of patients and although a high value was found in 10 of 14 untreated coeliacs, high values were also present in 24 of 96 non-coeliac, non-DH cases.

Food protein antibodies in jejunal fluids, series A

The CIA pattern was present in 13 of 14 newly-presenting coeliacs, and in 4 of the 5 new DH patients. Clinical, biopsy and immunological information on the two CIA negative cases are presented in Table VI.1; there were no unusual clinical features but it is of interest that the coeliac patient, who had classical enteropathy, also had a classical coeliac HLA phenotype, whereas this was not the case for the DH patient, whose jejunal mucosa was normal. Of the 22 coeliacs and one DH patient on GFD, the frequency of the CIA positive pattern was 69.5%, and 16 of the 98 non-coeliac patients (16.3%) were CIA positive. Results of assays of intestinal fluid IgA and IgM anti-gliadin antibodies are shown in Figures VI.3 and 4.

TABLE VI.1. CLINICAL, PATHOLOGICAL AND IMMUNOLOGICAL DETAILS OF TWO PATIENTS (COELIAC AND DH) ON NORMAL DIET, NEGATIVE FOR THE CIA PATTERN.

	MRS S.C.		MRS M.W.
DIAGNOSIS	Coeliac		D.Herpetiformis
DIET	Normal	GFD	Normal (low resid.)
AGE	44		49
INDICATION			
FOR JEJUNAL BIOPSY	Diarrhoea Iron def. Anaemia		D.Herpetiformis Variable bowel habit Asthma, eczema
BIOPSY HISTOLOGY	TVA	PVA	Normal
DISACCHARIDASES			Normal
IEL COUNT /100 enterocytes)	35	40	32
LACTULOSE/RHAMNOSE PERMEABILITY TEST	0.14*	0.034	0.02
SERUM: IgA-AGA	25.8*	8.7	4.8
IgG-AGA	51.0*	35.0	23.1
JEJUNAL FLUID:			
T.IgA (ng/ml)	11.5	19.1	32.7
T.IgM (ng/ml)	1.4	1.5	0.7
IgA-AGA	2.2	3.9	6.4
IgM-AGA	6.1	7.8	8.2
Other Abs	low	low	low
HLA TYPE:	A1,11;B8,57;BW4/6 DR3,7;DRW52/53;DQw2		A1,2;B44,35;BW4/6 DR2,4;DRW53;DQW1,3,7

*results abnormal;

normal values: permeability <0.04; IgA-AGA <20; IgG-AGA <40

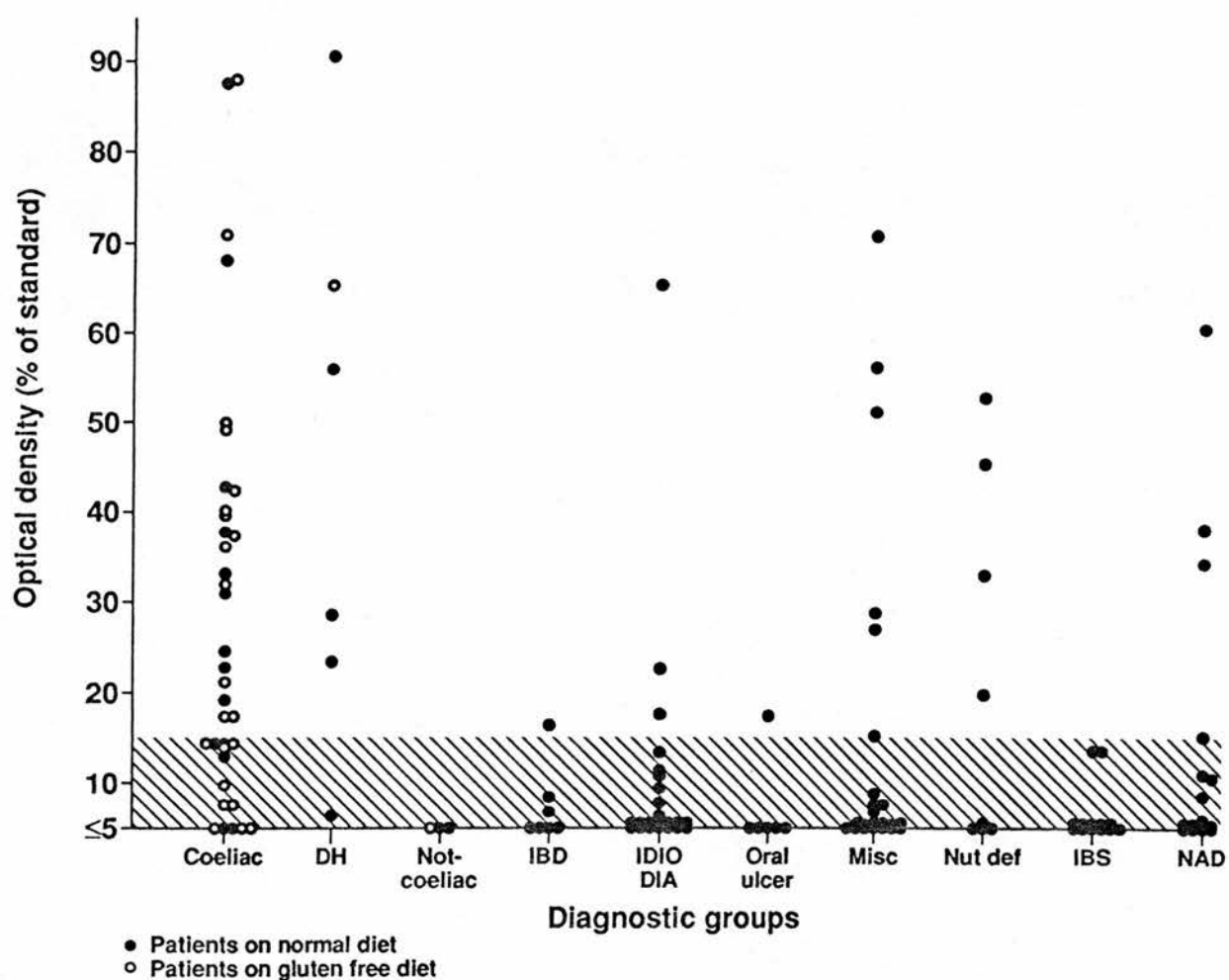


Figure VI.3: Jejunal fluid IgA antigliadin antibody levels in patients grouped by final diagnosis (Normal range is shaded).

Classification by intestinal antibody pattern, series A

The 140 patients were also subdivided by intestinal antibody findings into four groups: COE/CIA+ and COE/CIA- coeliacs and DH patients, who did (33 cases) or did not (9 cases) express the CIA pattern; OTH/CIA+ and OTH/CIA-, patients with other diagnoses who did (16 cases) or did not (82 cases) express the CIA pattern.

Immunoglobulin concentrations in jejunal fluids, series A

As shown in Table VI.2, concentrations of IgA and IgM in jejunal fluid were generally higher in coeliac and DH patients on a normal diet than in other diagnostic groups. When the patients were subdivided according to intestinal antibody patterns, it emerged that the two subsets with positive CIA patterns, COE/CIA+ and OTH/CIA+, had significantly higher concentrations of IgM than the CIA negative cases, irrespective of diagnosis. Total IgA concentrations were higher in the OTH/CIA+ group than in the other subsets.

Serum anti-gliadin antibodies and the intestinal antibody pattern, series A

There were no differences in the levels of serum

TABLE VI.2. IMMUNOGLOBULIN CONCENTRATIONS IN JEJUNAL FLUID.

		(n)	<u>TOTAL IgA</u>	<u>TOTAL IgM</u>
COELIAC	ND	14	224.5 , 244.7	25.8 , 29.6
	GFD	22	118.6 , 134.4	18.8 , 49.6
DH	ND	5	223.8 , 260	26.2 , 30.4
	GFD	1	132.4	14.5
NOT-COE	ND	1	39.4	8.9
	GFD	2	193.3	16.5
IBD		7	213.4 , 191.7	9.4 , 12
IDIO DIA		23	153.6 , 286.4	7.6 , 18.9
ORAL ULC		6	43.9 , 50.4	3.3 , 4
MISC		23	137.4 , 128.9	5.4 , 6.9
NUT DEF		8	260 , 348	24.7 , 41.7
IBS		13	102.3 , 161.9	1.9 , 1.5
NAD		15	181.8 , 206	2.9 , 3.6
COELIAC/ DH. ALL		42	167.5 , 194	21.5 , 42
CIA +VE		40	189.3 , 208	19.2 , 24.6
CIA -VE		2	78.3 , 86	2.3 , 1.8
OTHER DIAGNOSIS				
CIA +VE		16	426.6 , 335.7	25.4 , 33
CIA -VE		82	101.6 , 136.7	3.2 , 4.5

ND= normal diet

GFD= gluten-free diet

Data expressed as mean value, and standard deviation

antibodies between the CIA positive and CIA negative non-coeliac groups. In other words, high levels of serum anti-gliadin correlated with the presence or absence of gluten-sensitive enteropathy, but not with intestinal antibody levels.

Clinical details of CIA positive non-coeliac patients, series A

Details of diagnosis, reason for the biopsy and other studies performed in the group of 16 non-coeliac, non-DH cases in series A, positive for the intestinal antibody pattern, are presented in Table VI.3. In six cases the total IEL count was high, whereas in five of these other features of the biopsies were normal, one man had minor, non-specific abnormalities with short villi. Minor abnormalities of the mucosa were also present in two cases with normal IEL counts. The sugar permeability test was abnormal in only five cases, including the three with minor histological abnormalities.

Intestinal antibody pattern in series B

Jejunal fluid antibody levels were measured in a further 119 non-coeliac patients with normal jejunal biopsy histology. Positive CIA patterns were present in 2 of 8 "not-coeliacs", 2 of 14 patients with IBD, 4 of 12 with

TABLE VI.3. DEMOGRAPHIC DATA OF PATIENTS POSITIVE FOR THE COELIAC-LIKE ANTIBODY PATTERN IN JEJUNAL FLUID.

Case No	Age	Sex	Indication for Jejunal Biopsy	Final Diagnosis	Biopsy Histology	Tissue Disaccharidases	IEL count (/100 enterocytes)	Lact/ Rhamnose permeability ratio (normal <0.04)
1	20	f	Diarrhoea	Crohn's	Minor changes	Not done	27	0.134
2	24	f	Diarrhoea, abd.pain	Ulcerative colitis	Minor changes	Low (L,S)	15	0.240
3	67	m	Diarrhoea	Sclerosing cholangitis	Normal	Normal	21	Not done
4	19	f	Diarrhoea, wt loss	Idiopathic diarrhoea	Normal	Not done	80	0.012
5	28	f	Diarrhoea, wt loss	Idiopathic diarrhoea	Normal	Normal	27	0.011
6	56	m	Diarrhoea, IgA def.	Idiopathic diarrhoea	Normal	Normal	35	0.033
7	54	f	Diarrhoea	Microscopic colitis	Normal	Normal	46	0.032
8	43	m	Diarrhoea	Colonic polyps	Normal	Normal	22	0.020
9	32	f	Diarrhoea, anaemia	Pernicious anaemia	Normal	Normal	53	0.054
10	35	f	Anaemia	Iron deficiency	Normal	Normal	43	0.022
11	23	m	Anaemia	Iron deficiency	Normal	Normal	27	0.022
12	44	m	Diarrhoea, fatigue	Poor diet, somatiz/n disorder	Minor changes	Low (L)	73	0.032
13	29	f	Diarrhoea,abd.dist/n	Irritable bowel synd	Normal	Not done	22	0.008
14	19	f	Bloating,abd.dist/n	Nad	Normal	Normal	47	0.050
15	57	f	Bloating,abd.dist/n	Nad	Normal	Not done	20	0.045
16	25	f	Abdominal discomfort	Nad	Normal	Normal	18	0.036

idiopathic diarrhoea, 3 of 12 with mouth ulcers, 6 of 34 with various other diagnoses, 3 of 7 with nutrient deficiencies, one of 18 with irritable bowel syndrome and 3 of 14 with no abnormality detected.

Discussion and implications of these results

It has been shown previously that DH patients without mucosal involvement are also CIA positive (O'Mahony 1990a), and thus this pattern might provide a diagnostic test for latent coeliac disease in other situations. In this prospective study, 40 of 217 non-coeliac patients (series A + B) have a CIA positive pattern (20-30% of all referred for diagnostic biopsy); 14 of these also had other minor mucosal abnormalities (eg high IEL counts) and thus clearly have intestinal pathology at the mild end of the spectrum of gluten-sensitive enteropathy (Marsh 1989a, 1992). However, in contrast to DH latent coeliacs, most of these patients had symptoms suggestive of small bowel disease. Thus, quite apart from the theoretical interest in such patients, there is an important practical issue as to whether their diagnoses and management should be revised, and a GFD now prescribed.

It is now clear that CIA positive specimens also contain high concentrations of total IgM, in keeping with the

over-expression of IgM in the intestinal mucosa of coeliacs (Baklien 1977, Scott 1984, Dhesi 1984, Ciclitira 1986b, Wood 1987, Crabtree 1989a, Lavo 1992). This is not limited to food protein antibodies. In a recent investigation of intestinal immune responses to an enteric vaccine, coeliacs generated high levels of intestinal IgM antibodies to a bacterial antigen, Cholera toxin B subunit (O'Mahony 1990b).

From the assessment of the CIA expression by diagnostic groups, it is also interesting to point out that one of 14 newly-presenting coeliacs was CIA negative. Two of the eight cases of DH without enteropathy previously studied by us were also CIA negative (O'Mahony 1990a), as was one DH case without enteropathy in the present series. The fact that she lacked the HLA haplotype characteristic of coeliac disease (and was DR4 positive, as are other such atypical coeliacs) (Sachs 1986) may be important, and highlights the possibility that differences between patients in their intestinal expression of gluten-sensitivity, or in their mucosal immune responses, may have a genetic basis. It has been suggested that there may be separate genetic factors which regulate the capacity for gluten sensitization on the one hand, and a susceptibility to develop severe enteropathy in the expression of mucosal T cell mediated immunity on the other (Troncone & Ferguson 1991a).

Since this antibody pattern persists during strict gluten exclusion, the theory that it reflects a polyclonal response to lectin-like stimulation by gliadin cannot be sustained. Mucosal immunity is characterized by an overall down-regulation of isotypes other than IgA (Brandtzaeg 1991). The general increase in the IgM component of intestinal immunity which I have described could reflect a primary disorder of the B cell population (ie an abundance of "polyreactive" CD5+ B cells); an aberrant function of the T cell subsets which control the early stages of B cell development (ie suppressor T cell dysfunction) (Corazza 1986, Pignata 1985), or over-activity of local helper T cells (Troncone & Ferguson 1991a); or the existence of an unusual immunostimulant operating in mucosa.

The unusual up-regulation of IgM secretory responses may be an independent factor, perhaps genetically determined, reflecting a state of immunological hyper-responsiveness to many local stimuli, and relevant to the induction of a state of aberrant immunity to gluten. Similar findings have been obtained in some immuno-deficiencies (Geha 1979) with high serum IgM titres, signs of T cell activation such as high levels of soluble IL-2R in plasma, and high number of HLA-DR+ cells; and other autoimmune phenomena which often have a genetic basis.

Non-coeliac CIA positive patients seem to have a dysbalance of lamina propria plasma cells, manifested by an increased number of IgM-containing cells. Alternatively, an immunoregulatory T cell-related disturbance may determine an altered pattern of cytokine production which might lead to the persistence of some mucosal abnormalities; eg polyclonal IgM secretion by B cells without antigen, or terminal differentiation of IgM+ B cells, induced by IL-5 and IL-6.

Antigen-related terminal differentiation and expansion of IgM and IgA B cells appear to be normal. Trans-epithelial immunoglobulin transport is also normal (Baklien 1977), indeed the production of SC by epithelial cells is increased in coeliac disease (Scott 1981). An abnormally high capacity for transport of IgM and IgA across the epithelium might produce the CIA pattern. However, I have examined the molecular forms of jejunal fluid IgA (with and without SC) and found no differences between CIA positive and CIA negative specimens (Chapter VII). It has been suggested that the affinity for the SC of IgM is much higher than dimeric IgA. Thus over-production of IgM in the lamina propria may partially block trans-epithelial IgA transport - an explanation for the presence of dimeric IgA in the serum in coeliac disease (Troncone 1991b).

According to the new putative roles suggested for IgA in mucosal immunity (Mazanec 1993), this immunoglobulin may be involved in the excretion of immune-complexes (IC) and other potentially damaging products from intestinal mucosa. However, IgM-IC are not so easily removed, and high titers of secretory IgM antibodies, found in CIA positive patients, may form large IC with antigen, more difficult to be excreted into the lumen, contributing to maintaining the antigen for a longer time in the lamina propria, and therefore increasing the possibility of antigen stimulation. Moreover, IgM may activate complement, leading to a cascade of other inflammatory reactions, and the immuno-complexes formed may reach the capillaries and the systemic circulation.

Positivity for the CIA pattern is shown by almost 20% of patients referred for diagnostic biopsy, and its frequency is similar amongst different diagnostic groups. Some of these patients had gut symptoms, but there is a proportion in whom no abnormality was detected. Screening for jejunal fluid antibodies is a simple procedure that may identify patients susceptible of being followed up and studied for the expression of other markers of potential coeliac disease.

CHAPTER VII

JEJUNAL MUCOSA STUDIES IN SYMPTOMATIC PATIENTS REFERRED AT THE G.I. UNIT

Introduction

In the course of the study of secretory antibody patterns in non-coeliac patients referred for diagnostic jejunal biopsy, a report was published which showed a high $\tau\delta$ IEL count in an histologically normal stored biopsy of an individual who later developed an enteropathy (Maki 1991a). Looking for other putative markers of latent coeliac disease in the same non-coeliac population, I thus set up arrangements to obtain material suitable for frozen sections from jejunal biopsies collected at the time of fluid sampling, in order to address the question of whether non-coeliac CIA positive patients also had a high density of $\tau\delta$ + IELs.

Given that the reason for investigating these patients was the existence of small bowel symptoms and therefore the suspicion of coeliac disease; the question arose of whether the expression of these mucosal abnormalities (eg

high $\tau\delta$ + IEL counts) might occur in the non-coeliac population as a whole. To address this, I also collected duodenal biopsies from an unselected group of patients attending for upper endoscopy, to study $\tau\delta$ + cells in epithelium.

It was of practical interest was to compare two techniques for counting IELs, the conventional count expressed as a total IEL number per 100 enterocytes, using formalin-fixed, H & E paraffin sections of jejunal mucosa; and CD3+ and $\tau\delta$ + IEL analysis of frozen sections of jejunal mucosa stained by appropriate monoclonal antibodies.

I assessed other possible relationships with the CIA pattern, and the co-expression of potential markers in particular cases, eg abnormal intestinal permeability test, serum antibodies, etc. Positive ARA/AEm antibodies of IgA class have been reported in latent coeliac patients (Maki 1990), and in collaboration with this author, all patients in series C and other cases with different patterns of expression of potential markers, have been assessed for these serum antibodies.

Intraepithelial lymphocyte counts, series A and B

With the technique used the normal range is 10-40 IEL per

100 villus enterocytes, and virtually all untreated coeliacs have abnormally high counts within the flat surface epithelium (eg results in our studies of four separate groups of patients have ranged from 44-180 IEL per 100 enterocytes) (Ferguson & Murray 1971, 1976; Ziegler & Ferguson 1984, Ferguson & Ziegler 1986). Results for biopsies from patients in series A and B are shown in Figure VII.1. Abnormally high counts were present in one (CIA positive) of 7 treated coeliacs, in 3 of the 4 DH patients (all three were CIA positive), and in 15 patients with other diagnoses. Fourteen of these were CIA positive cases; IEL count was high in only one CIA negative non-coeliac patient, a woman with colonic Crohn's disease.

Reference values for $\gamma\delta$ + IEL counts, series C and D

A subset of 34 jejunal biopsy patients, immunologically normal, was used to establish reference ranges for jejunal CD3+ IEL cells and $\gamma\delta$ + IEL counts per mm epithelium. There were 16 with IBS, 14 with trivial or transient disorders and 4 in whom no abnormality had been detected. Reference values for the upper limit of normal (mean + 2SD) thus obtained were 67.5 CD3+ IEL per mm epithelium and 5.5 $\gamma\delta$ + IEL per mm epithelium, respectively. An example of frozen sections stained for

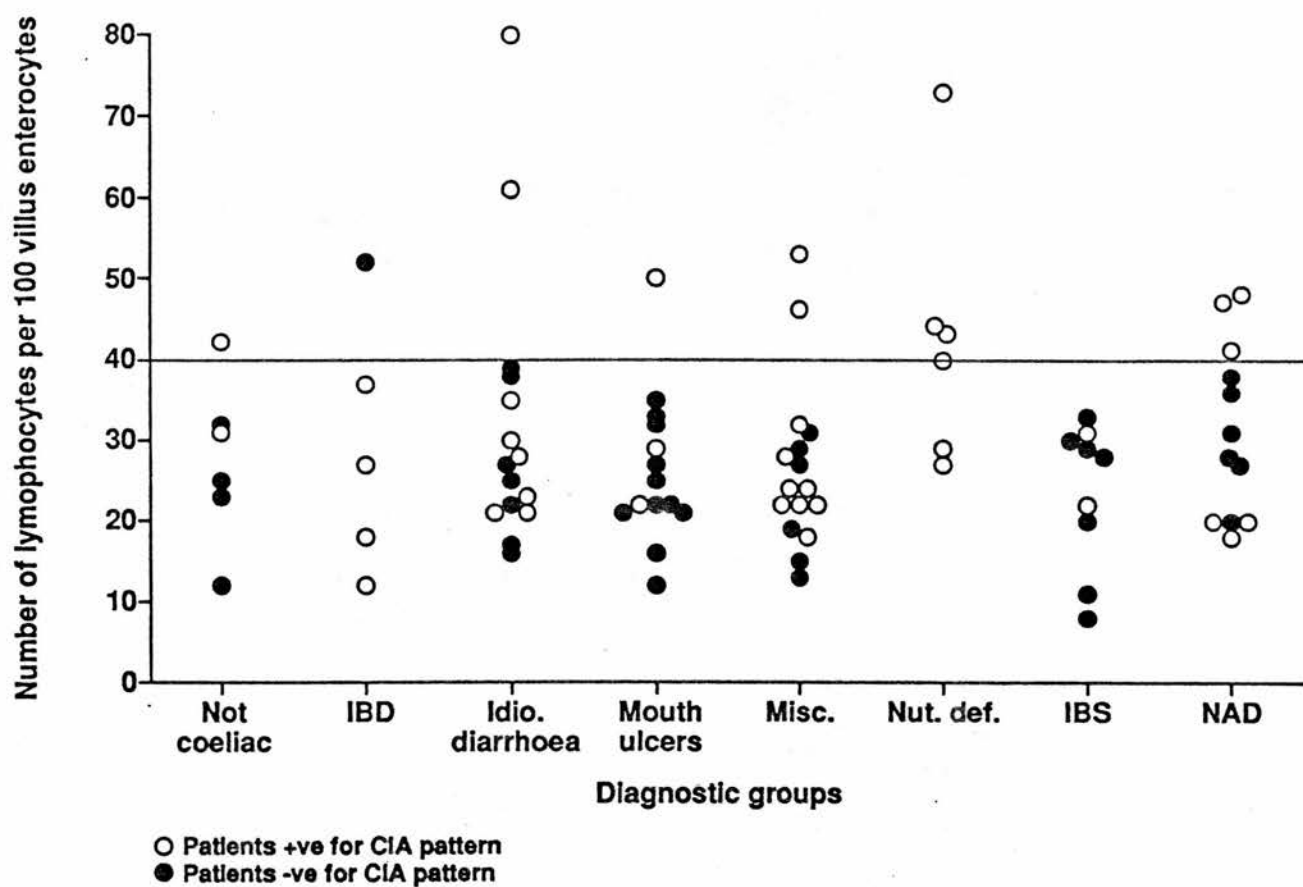
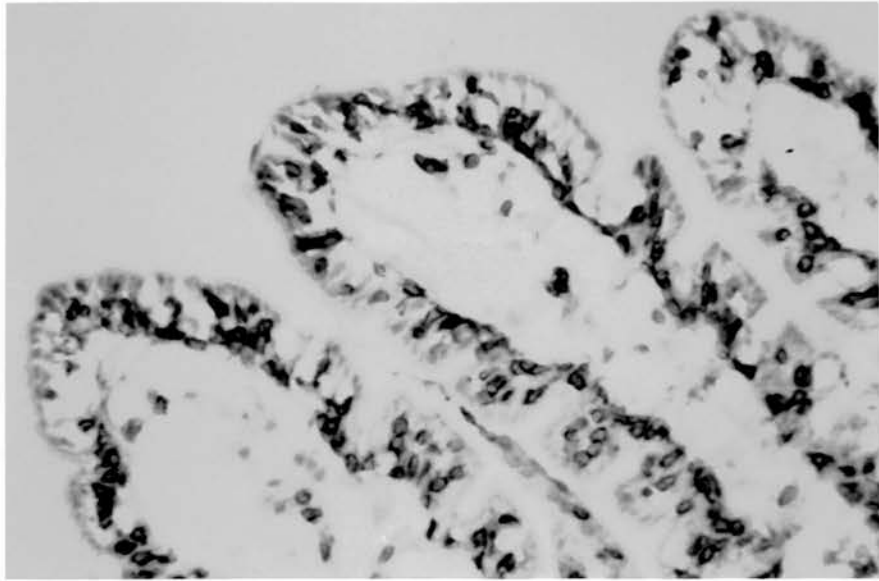
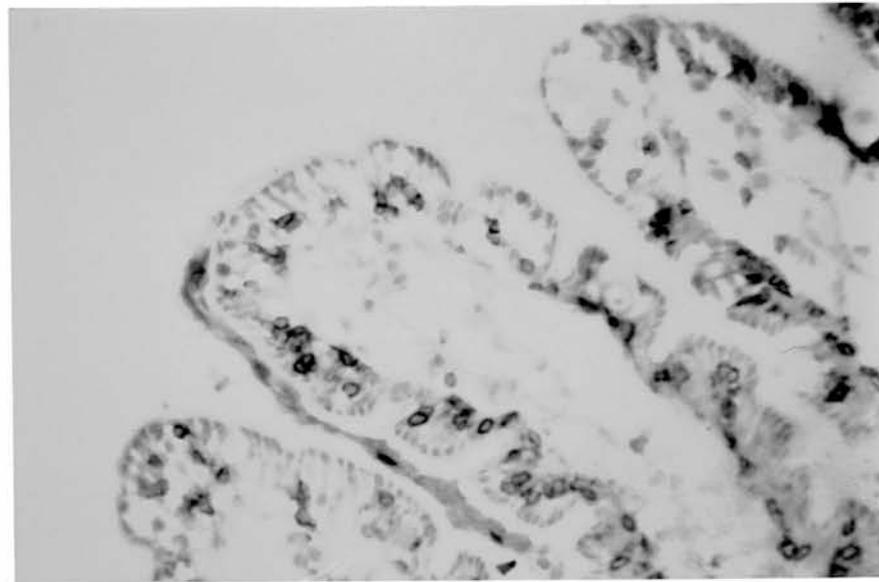


Figure VII.1: Intraepithelial lymphocyte counts in jejunal biopsies from 94 patients, reported as normal or with minor histological changes (Normal range 10-40 lymphocytes per 100 villus enterocytes).



a)



b)

Figure VII.2: Serial cryostat sections of jejunum from a non-coeliac patient with high total IEL count. Immunoperoxidase staining of intra-epithelial lymphocytes with antibodies to CD3 (Figure 9a) and to TCR $\delta 1$ (Figure 9b).

CD3+ and $\tau\delta$ + IEL is shown in Figure VII.2. The results for $\tau\delta$ + IEL counts are presented as the unmodified count per unit length of surface villus epithelium, taking counts >5.5 $\tau\delta$ + cells per mm of epithelium as abnormally high (Table VII.1). Fourteen of the biopsies from coeliacs had abnormally high $\tau\delta$ + IEL counts. The remaining patient (normal mucosa, on a GFD, with no unusual clinical features) had a normal count; his data is presented separately from the other cases.

There were significant differences between the reference values obtained for CD3+ cells in duodenal and jejunal specimens (Table VII.1), with values for duodenum (mean 19.4 CD3+ per mm epithelium) significantly lower ($p<0.001$) than for jejunum. However, $\tau\delta$ + cell counts per mm of epithelium were similar in the two sites. There was one endoscopy patient (a woman with an uncomplicated gastric ulcer) who had a strikingly high $\tau\delta$ + cell count at 24 per mm of epithelium; in other respects her duodenal biopsy was normal.

Comparison of CD3+ and Total IEL counts, series C and D

The material examined from the 77 non-coeliac patients in the present series allows a comparison of the two techniques of IEL counts: in H & E stained sections, using a conventional light microscope, and with results

TABLE VII.1. VALUES FOR CD3+ and TCR $\tau\delta$ + CELL COUNTS IN VILLOUS EPITHELIUM

	CD3+ CELLS PER MM	TCR τ/δ CELLS PER MM
Coeliac patients (n=15)	+69.2 , 32.9 (31.9-120)	+21.5 , 16.7 (5.5-66)
Atypical case	42.2	0.2
Control group Jejunal biopsy patients (n=77)	*33.7 , 16.9 (6-68.7)	1.5 , 2 (0-7.4)
Duodenal biopsy patients (n=26)	19.4 , 11.4 (2.3-45.9)	1.5 , 4.7 (0-24.4)
Excluding atypical case	18.4 , 10.3 (2.3-44.1)	0.6 , 0.6 (0-2.5)

* CD3+ cell counts were higher in the control group ($p < 0.001$) than in duodenal biopsy patients.

+ Coeliac patients had higher CD3+ and TCR $\tau\delta$ + counts ($p < 0.001$) than both controls and duodenal biopsy patients.

Data expressed as the mean, standard deviation (range values)

expressed as number of IEL per 100 enterocytes; and in frozen sections and image analysis where counts are expressed as CD3+ cells per mm of epithelium. As shown in Figure VII.3, there was a highly significant correlation between IEL counts in paraffin sections and CD3+ cell counts in frozen sections ($r=0.771$, $p<0.001$). All cases with high CD3+ cell counts had high IELs, but there were 7 cases with high IEL count, normal CD3+ cell count, suggesting that a non-T subset of lymphocytes may be present in the epithelium of these patients.

Positive markers of potential coeliac disease, series C

Results for these 77 non-coeliac patients subdivided into diagnostic groups, are summarized in Table VII.2. The CIA pattern of jejunal fluid antibodies was present in 21 patients. There were 9 patients with high counts of $\gamma\delta$ + IEL in the villus epithelium, and conventional IEL counts were high in 13 cases. High titers of serum IgA anti-gliadin antibodies were present in 12 patients, with no obvious relationship with diagnostic groups or the other markers, except for the sugar permeability test ($p<0.05$), which was abnormal in 9 cases, mainly those with mild abnormalities of jejunal pathology or with IBD.

Associations between the CIA pattern, high $\gamma\delta$ + cell counts and high total IEL counts are illustrated

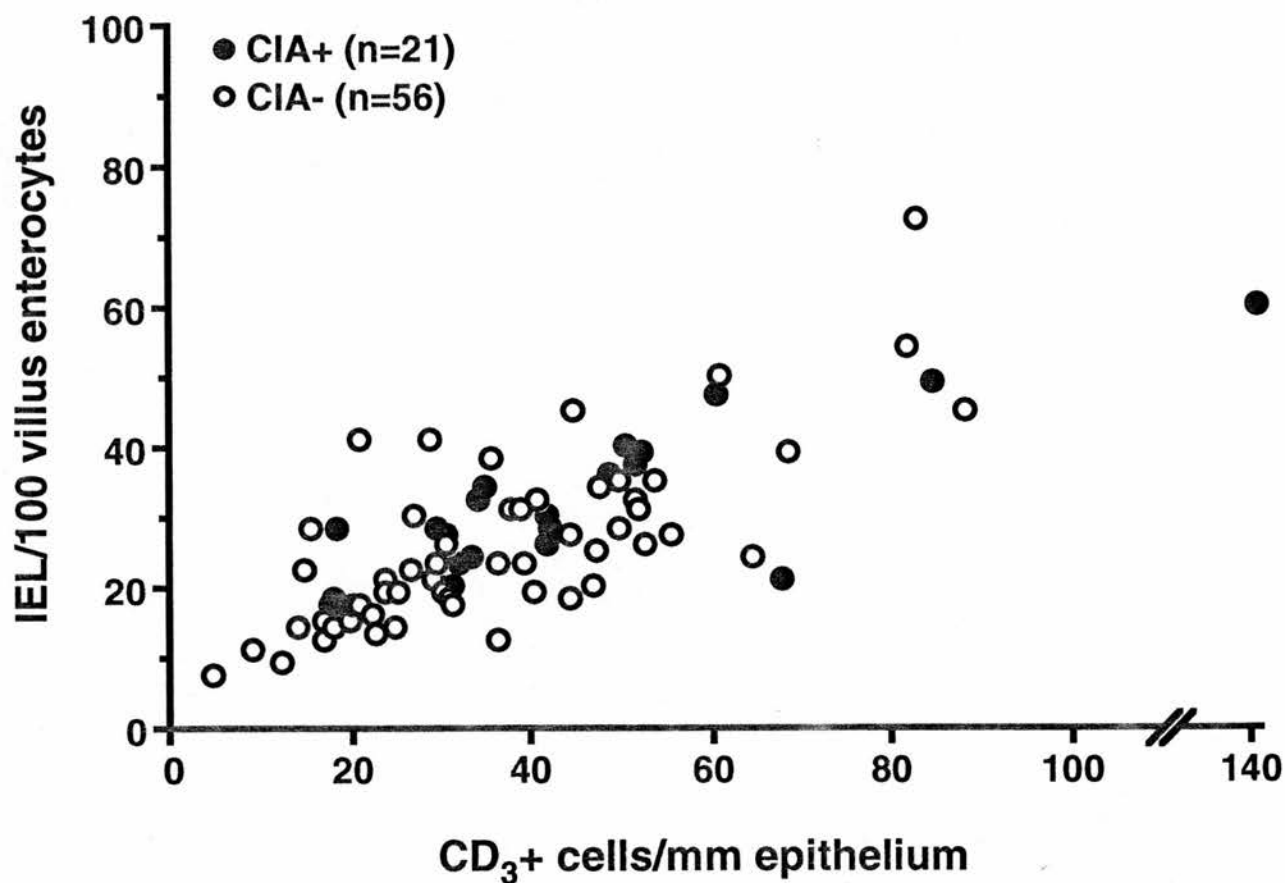


Figure VII.3: Intra-epithelial lymphocyte counts in 77 pairs of jejunal biopsy specimens. Total IEL counts in paraffin sections are expressed as IEL per 100 villus enterocytes; CD3+ cell counts in frozen sections are expressed as cells per mm length of villus epithelium.

TABLE VII.2. DIAGNOSTIC GROUPS: NUMBERS OF CASES POSITIVE FOR CANDIDATE MARKERS OF LATENT COELIAC DISEASE.

FINAL DIAGNOSIS	(n)	CIA+ CASES	HIGH τ/δ COUNTS	HIGH IEL COUNTS	ABN.L/R TEST	HIGH SERUM AGA LEVELS
NOT COE	4	2	1	1		
FH	4	1				1
IBD	6	2	1		4	2
DIA IDIO	14	4	3	4	1	2
ORAL ULC	4	2	1	2	1	1
MISC	19	4		2	2	3
NUT DEF	4	2	1	1		
IBS	16	3	2		1	2
NAD	6	1		3		1
COE. N diet	1	1	1	not done	1	1
Glu Ch	1	1	1	not done	1	1
GFD	13	8	12	not done	6	4

ABN.L/R=abnormal lactulose/rhamnose permeability test

diagrammatically in Figure VII.4. High $\tau\delta$ + IEL counts were found in 6 of the 21 CIA positive patients, but only in 2 of 56 CIA negative ($p<0.001$); there were no significant associations in any other marker combinations. A high total IEL count was more frequent in CIA positive patients (5 of 21), than in patients negative for the CIA pattern (8 of 56), but the difference was not significant.

There were 31 patients with one or more positive result, but in 19 only a single test was abnormal. Of particular interest are patients with three parameters positive: CIA pattern, $\tau\delta$ + IEL count and total IEL count. Two of the three have been shown to be gluten-sensitive after a trial of a GFD (see Chapter X). In one case, the diarrhoea resolved within 2 weeks of gluten restriction and a normal IEL count was found in a biopsy taken 2 months later. In the second case, she noticed some improvement of her recurrent aphthous ulceration but took the diet for only five weeks. Several months later, when a high count of $\tau\delta$ + IEL was found in a stored biopsy specimen, she agreed to take an extra 10 g of gluten daily for a month and had another jejunal biopsy. Pathological examination revealed STVA.

Anti-reticulin and anti-endomysium antibodies

A description of the patients studied is shown in Table VII.3. Serum antibody levels were positive in 2 of the 6 coeliacs studied (both on a gluten-containing diet, and showing PVA). Amongst non-coeliac patients, there were 6 positive cases: two had very low levels (eczema, and Crohn's disease) and both were negative for other putative markers. A third case had only high ARA levels, and borderline counts for both total IEL and $\gamma\delta$ + IELs (39 per 100 enterocytes, and 6.5 cells per mm of epithelium). Of the other 3 cases, two (idiopathic diarrhoea, recurrent mouth ulcers) were positive for the full set of markers of potential coeliac disease, CIA pattern, high counts of total and $\gamma\delta$ + IELs. Both responded clinically to a trial of GFD, and one is the case who developed an enteropathy after adding extra gluten to her already normal diet. The third non-coeliac positive case was previously incorrectly diagnosed as having coeliac disease (without a positive biopsy), and had low $\gamma\delta$ + IEL counts.

Discussion and implications of these results

I have presented the results of a potential way of identifying patients who may have latent coeliac disease,

by the expression of a characteristic CIA pattern in jejunal fluid (see Chapter VI). It was shown that 14 of the 40 non-coeliac CIA positive patients also had a high IEL count, and therefore they have intestinal pathology at the mild end of the spectrum of gluten-sensitive enteropathy (Marsh 1989a, 1992). Latent (or low-grade) coeliac disease may also be recognized by studies of TCR $\gamma\delta$ expression by IEL, which have been found to be increased in DH patients and in a case of latent coeliac disease detected during a family study (Savilahti 1990, 1992; Maki 1991a). Up to 30% of coeliac relatives with normal biopsy histology had high $\gamma\delta$ IEL counts, and the number of positive $\gamma\delta$ cells is associated with the HLA-DQ α/β gene markers in a dose dependent manner (Holm 1992, 1993b). Healthy first degree relatives of coeliac patients may have ARA and AEm antibodies (Maki 1990); and also changes in intestinal permeability (Bjarnason 1983).

These results, together with in-vivo gluten challenge studies in coeliac patients (see Chapter X), indicate that the expression of gluten hypersensitivity as enteropathy may be minimal, measurable only if a count of IEL is performed. An accepted name for this type of pathology is needed, such as "high IEL mild enteropathy" (Ferguson 1993). This must be clearly differentiated from low-grade or other forms of non-coeliac enteropathy. However, the presence of the CIA pattern seem to be an

TABLE VII.3. DEMOGRAPHIC DATA OF PATIENTS STUDIED FOR SERUM ANTI-RETICULIN (ARA) AND ANTI-ENDOMYSIUM ANTIBODIES (AEm).

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD		HISTOLOGY NORMAL MINOR CHANGES		PTVA
COELIAC	5	42 (16-57)	3/2	2	3	2		3
NOT COE	8	31 (17-64)	6/2	5	3	7	1	
POT COE	2	58	1/1	2		2		
IBD	6	38.5 (23-50)	4/2	6		6		
DIA IDIO	17	33 (19-70)	9/8	16	1	13	4	
ORAL ULC	14	32 (19-57)	3/1	4		2	2	
MISC	19	41.5 (26-68)	12/7	19		16	3	
NUT DEF	5	26 (17-34)	5/-	4	1	3	2	
IBS	17	36 (21-71)	13/4	17		16	1	
NAD	7	23.5 (15-55)	5/2	7		5	2	

NOT-COE= previous diagnosis on inadequate criteria

POT COE= potential coeliacs on follow up studies

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

even more sensitive index of mucosal DTH expression than is IEL count.

Some of the cases in the literature, conforming to the present definition of latent coeliac disease, will, on review of biopsy pathology, be found to demonstrate high IEL mild enteropathy, thus falling within the "continuum" concept of gluten-sensitive enteropathy (Marsh 1992). However, there are already published and unpublished cases in which the total IEL count in the original biopsy was normal. Not all such patients are asymptomatic, so the word "latent" may in any event be inappropriate in relation to the full clinical expression of gluten sensitivity.

In humans, the finding of a high IEL count in an otherwise morphologically normal mucosa may reflect a state of T cell activation, probably antigen-driven. Morphometric studies have shown that this is an early expression of gluten-sensitivity (Marsh 1980, Leigh 1985), that may follow lamina propria T cell activation (Monk 1988). In some cases, this minimal lesion may progress to a fully-expressed enteropathy. In first-degree relatives, a correlation has been found between IEL counts and the positivity for HLA-DR3, but this does not occur amongst coeliac patients (Holm 1993a).

The expression of the TCR $\gamma\delta$ by IELs in coeliac disease may be genetically determined (Savilahti 1992; Holm 1992, 1993b), but local factors might also be involved, perhaps after being triggered by gluten (Kluin 1991, Deusch 1991). These cells have a limited capacity for antigen recognition, and no gluten specificity has yet been reported (Haltensen 1989, Rust 1992, De Libero 1993). It is very unlikely that $\gamma\delta$ cells play a role in mucosal damage, because high $\gamma\delta$ + IEL counts have been found in non-coeliac patients with a normal biopsy histology, and in a case of latent coeliac disease before the development of enteropathy (Maki 1991a).

In much of the published work on latent coeliac disease, and on the spectrum of pathological expression of gluten sensitivity, IEL counts have been performed in H&E stained sections, using a conventional light microscope. In the future, if frozen sections and image analysis are necessary to count $\gamma\delta$ + IEL, it may be preferable to count CD3+ cells per mm as an alternative to total IEL per 100 enterocytes. A direct comparison of these two techniques presented in this chapter (Figure VII.3), shows a highly significant correlation between IEL counts in paraffin sections and CD3+ cell counts in frozen sections. All cases with high CD3+ cell counts had high IELs, but there were 7 cases with high IEL counts and a normal CD3+ cell count, suggesting that a non-T cell subset may be present

in the epithelium of these patients.

A high total IEL count, in an H&E stained section, may be due to expansion of one or more of the three main subsets of lymphocytes within the villus epithelium of the small intestine: (i) CD3+ IEL (T cells) which utilize the $\alpha\beta$ TCR; numbers of these rise and fall in coeliac patients with gluten ingestion and exclusion; (ii) CD3+ IEL (T cells) which utilize the $\gamma\delta$ TCR; generally, counts of these cells are high in coeliac patients, irrespective of diet; (iii) a small proportion of IEL are CD3- cells, with no T cell receptors; their nature, in man, is uncertain. This non-T/non-B IEL (atypical) subset may occasionally be expanded, as shown above. Further patients will need to be studied in order to establish the clinical significance of this finding.

It has been shown that a CIA positive pattern is significantly associated with high $\gamma\delta$ + IEL counts, and also (though not statistically significant) with high total IEL count. I have also confirmed the previous finding that patients with subtle immune changes in gut humoral immunity have entirely normal levels of serum IgA anti-gliadin antibody (O'Mahony 1991a, Arranz & Ferguson 1993). A positive sugar permeability test is independent of the other features, and it is mainly abnormal in patients with inflammatory bowel disease and alcohol or

NSAID-related problems.

The CIA pattern and a high $\gamma\delta$ + IEL count occur independently in some non-coeliacs, but also co-exist more frequently than expected by chance. These phenomena may be due to separate, intrinsic, genetically determined aberration of the constituent lymphocyte populations of the mucosal immune system. They may be expressed in all phases of intestinal immune activation, but it is equally possible that aberrations occur only as part of the expression of immune responses and inflammation in the gut. Whatever the underlying mechanism, the repertoire selected from the full range of potential immune cells and molecules includes intestinal IgM antibodies and $\gamma\delta$ + IEL in coeliacs, whereas these are not utilized in most non-coeliacs.

Other groups have reported the results for $\gamma\delta$ + IEL counts in frozen sections as a percentage of the total number of CD3+ IEL (Spencer 1989a, 1991; Tredjosiewicz 1989, 1991; Kluin 1991). However, I found that method produced spuriously high, abnormal values in a few cases with very low total CD3+ cell counts (presumable with deficiency of $\alpha\beta$ IEL rather than excess of $\gamma\delta$ cells); and therefore the results are presented as the unmodified count of $\gamma\delta$ + IEL per unit length of surface villus epithelium.

The differences in the reference values obtained for CD3+ cells in duodenal and jejunal specimens ($\gamma\delta$ + IEL counts per mm of tissue were similar in the two sites), may reflect a true difference between these tissues, but alternatively may be explained by the fact that duodenal biopsy patients were significantly older than the reference jejunal biopsy group (I have previously reported low IEL counts as a feature of human immunosenescence (Arranz 1992)).

With the currently available range of tests, studies of a single putative marker cannot be expected to identify all cases of latent coeliac disease. The two best indices are a positive CIA pattern and high $\gamma\delta$ count. However these are not present even in all classical coeliacs. I found one of 14 untreated coeliacs and one of 6 DH patients in series A were CIA negative (Arranz & Ferguson 1993), and one otherwise completely typical coeliac man in series C (who also has insulin-dependent diabetes) had $\gamma\delta$ + IELs undetected. Although logistically difficult, the identification of potential coeliacs still requires studies of several candidate markers, using jejunal fluid for ELISA studies, and frozen sections of a mucosal biopsy for T cell receptor staining.

For prospective studies of candidate latent coeliacs, such as those with high IEL count, positive CIA pattern,

high TCR $\gamma\delta$ expression of IEL, relatives of coeliacs, IgA deficiency, a more generally applicable expression is needed, for instance the term **potential coeliac disease** (Ferguson 1992).

The combined assessment of the CIA pattern and several other immunological and functional tests shows that these markers will only identify potential coeliac cases, and gluten sensitivity still requires clinical monitoring on a GFD and after a gluten challenge. It is important to emphasize that a high density of villus IEL (as assessed by differential counts in the present study) may be the only pathological expression of gluten sensitivity in coeliac patients (Marsh 1989a), defining a mild but significant enteropathy, rather than a truly latent condition. Thus a positive result for total IEL count may have different implications than positive findings for the other parameters examined.

CHAPTER VIII

STUDIES OF LAMINA PROPRIA PLASMA CELLS

Introduction

Markers of potential coeliac disease may be integrated in the spectrum of gluten-sensitivity in two ways, representing either the primary mucosal abnormality, which predisposes to the existence of gluten sensitivity when other predisposing factors occur; or alternatively they may be subtle evidence of the existence of an on-going hypersensitivity reaction activated by local immuno-stimulating factors, or by other mechanisms. Though I pursued the latter hypothesis, the first possibility was also explored by counting lamina propria IgA+ and IgM+ containing plasma cells in jejunal biopsy sections. The aim was to assess whether total IgA and IgM concentrations, or specific class antibodies, found in jejunal fluid specimens, were due to differences in the total mucosal B cell population, or to differential transport across the epithelium.

I compared two groups of "probably normal" non-coeliac patients with minor GI symptoms and a variety of

disorders, who were distinguished by the expression of the CIA pattern in their jejunal fluid. These comprised 15 CIA positive patients, 10 of whom had a normal biopsy histology and 5 had a high IEL count; and 14 CIA negative patients, all with a morphologically normal biopsy including a normal IEL count. I also included a group of 12 coeliac patients as an abnormal group, known to have a high number of lamina propria IgM+ plasma cells as well as a CIA positive pattern. All sections studied here correspond to patient included in series A, and they are described in Table VIII.1.

Lamina propria cell counts in jejunal biopsies

Non-coeliac CIA positive patients had a higher number of lamina propria IgM+ plasma cells ($p < 0.05$) when compared to patients negative for the CIA pattern. Untreated coeliac patients had significantly higher counts of IgM+ plasma cells compared with both non-coeliac patient groups, either positive or negative for the CIA pattern ($p < 0.01$). However, when these non-coeliac groups were compared with treated coeliacs, the difference was not statistically significant (Figure VIII.1).

Untreated coeliacs had a higher number of IgA plasma cells when compared with non-coeliac patients positive and negative for the CIA pattern ($p < 0.01$). No

TABLE VIII.1. DEMOGRAPHIC DATA OF PATIENTS STUDIED FOR LAMINA PROPRIA PLASMA CELL COUNTS IN JEJUNAL BIOPSIES.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD	HISTOLOGY NORMAL MINOR PTVA CHANGES
COELIAC					
Untreated	6	44 (23-76)	4/2	6	6
Treated	6	38.5 (14-82)	4/2	6	2 2 2
POT COE	2	58	1/1	2	2
IBD	2	22.5 (20-25)	2/-	2	2
DIA IDIO	5	48 (26-67)	3/2	5	5
ORAL ULC	3	23 (19-26)	3/-	3	3
MISC	6	53 (33-76)	5/1	6	5 1
NUT DEF	3	34 (24-44)	2/1	3	1 2
IBS	3	40 (30-46)	3/-	3	2 1
NAD	7	35 (20-57)	5/2	7	6 1

NOT-COE= previous diagnosis on inadequate criteria

POT COE= potential coeliacs on follow up studies

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

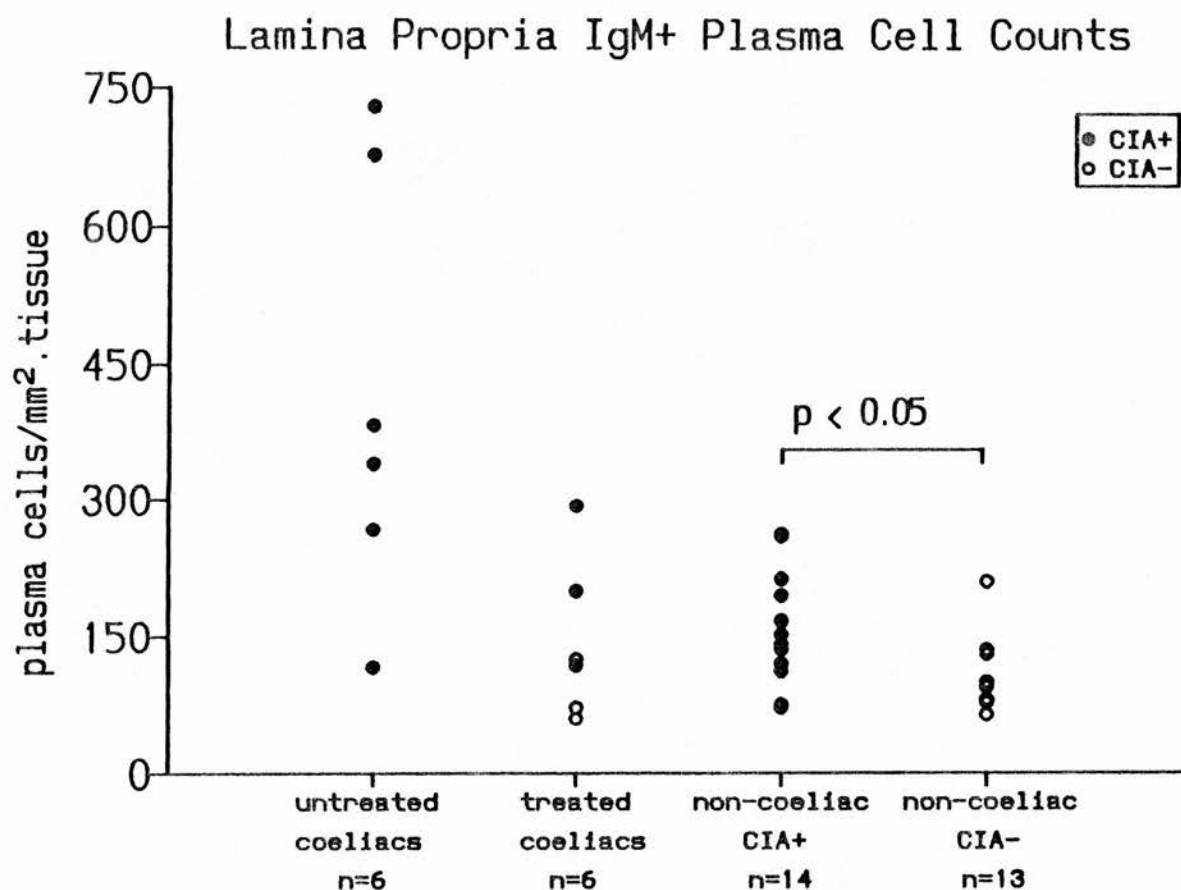


Figure VIII.1: Lamina propria IgM+ plasma cell counts per square mm of tissue in treated (n=6) and untreated (n=6) coeliacs, and non-coeliac patients positive (n=14) and negative (n=13) for the CIA pattern.

statistically significant differences were found between non-coeliac patient groups, or when these groups were compared to treated coeliacs (Figure VIII.2). Results for the different Ig+ class plasma cell counts and patient groups are shown in Table VIII.2.

Lamina propria IgM+ and IgA+ plasma cell counts do not correlate with concentrations of IgA and IgM-class specific immuno-globulins in jejunal fluid specimens.

Discussion and implications of these results

An over-expression of IgM generally in the intestinal mucosa has been reported in coeliacs (Baklien 1977, Scott 1984, Dhesi 1984, Ciclitira 1986b, Wood 1987, Crabtree 1989a, Lavo 1992), and also in non-coeliac CIA positive patients, as shown by the study of jejunal fluid immunoglobulins and antibodies (see Chapter VI), and counts of lamina propria plasma cells described in this chapter. Antibody responses are not restricted to gliadin, and high levels of intestinal IgM antibodies to Cholera toxin B subunit have been also found in coeliacs (O'Mahony 1990b).

In the present chapter, I have assessed the hypothesis that the general increase in the IgM component of intestinal immunity might reflect a primary disorder of B

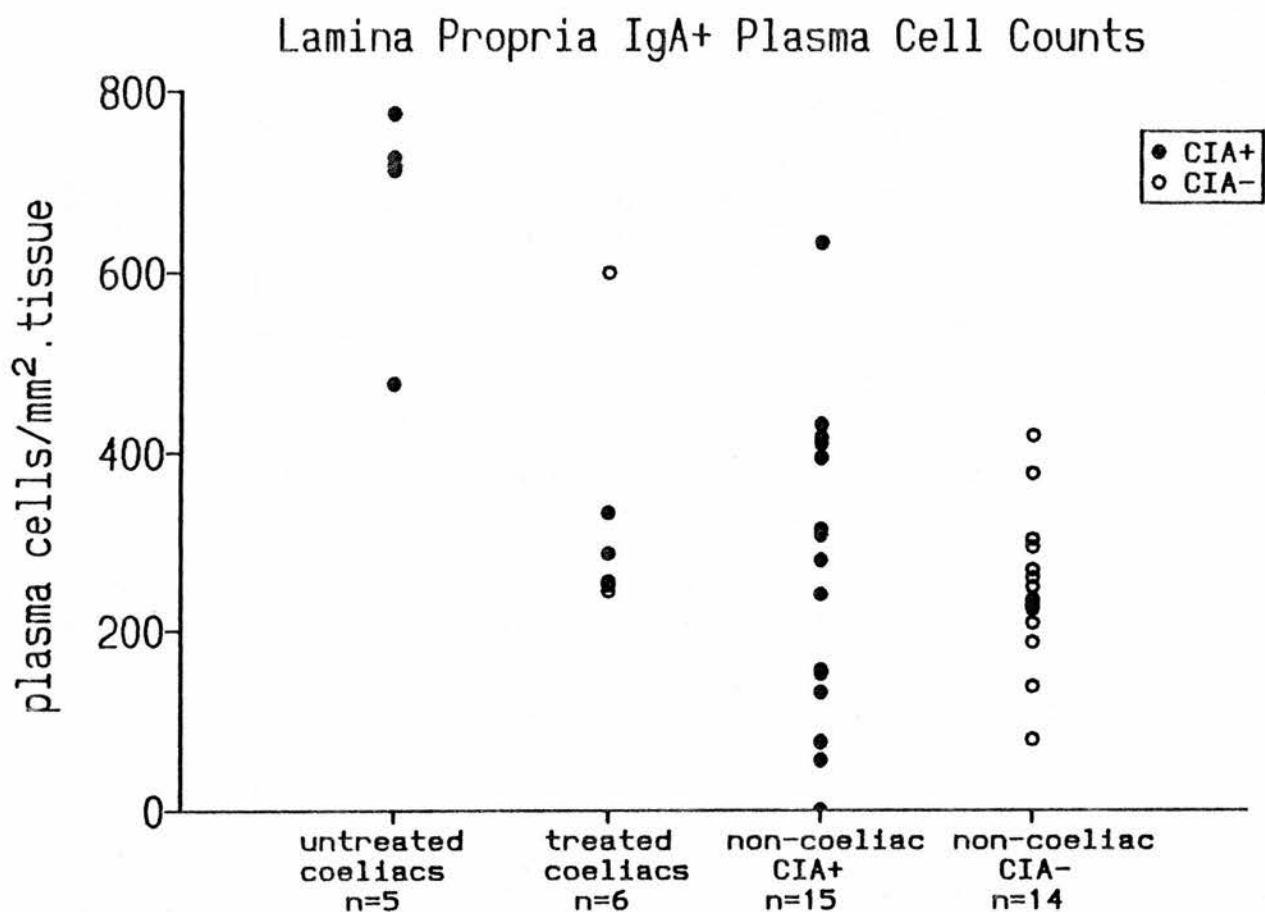


Figure VIII.2: Lamina propria IgA+ plasma cell counts per square mm of tissue in treated (n=5) and untreated (n=6) coeliacs, and non-coeliac patients positive (n=14) and negative (n=13) for the CIA pattern.

TABLE VIII.2. NUMBER OF LAMINA PROPRIA PLASMA CELLS PER SQUARE MILLIMETER OF JEJUNAL BIOPSY TISSUE

	(n)	IgA+ PLASMA CELLS (/mm ² LP tissue)	IgM+ PLASMA CELLS (/mm ² LP tissue)
NON-COELIAC PATIENTS			
CIA positive	(15)	267 , 174	#157 , 65
CIA negative	(14)	250 , 85	106 , 36
COELIAC PATIENTS			
Normal diet	(6)	*682 , 118	++419 , 239
GFD	(6)	*330 , 137	*145 , 88

* Coeliac patients, both on a normal diet and on GFD, had higher number of IgA+ and IgM+ plasma cells ($p < 0.001$) than non-coeliac patients positive and negative for the CIA pattern.

+ Coeliac patients on a normal diet had higher number of IgM+ plasma cells ($p < 0.05$) than coeliacs on GFD.

Non-coeliac patients positive for the CIA pattern had higher IgM+ plasma cells ($p < 0.05$) than non-coeliacs CIA negative.

Data expressed as the mean value and standard deviation

cells in the lamina propria (eg an abundance of poly-reactive CD5+ B cells). This population of "abnormal" cells might determine the production of polyclonal low-affinity IgM antibodies. If the increased proportion of IgM produced cannot compete successfully with dimeric IgA for the SC, the molecules are susceptible to rapid cleavage by proteolytic enzymes, and this susceptibility is increased by the lack of stability of the molecule lacking SC.

An alternative explanation for the increased up-regulation of IgM responses, namely that the "abnormality" may be due to the existence of an aberrant function of T cell subsets which control the early stages of B cell development, and probably manifested by an abnormal pattern of mucosal activation or cytokine production, is assessed in Chapter IX.

The unusual up-regulation of IgM secretory responses in CIA positive patients may be genetically determined, reflecting a state of immunological hyper-responsiveness to many local stimuli, and relevant to the induction of a state of aberrant immunity to gluten. In addition, an immunoregulatory T cell disturbance may also determine an altered pattern of cytokine synthesis that might lead to the persistence of some mucosal abnormalities, eg polyclonal IgM secretion by B cells without antigen, or

terminal differentiation of IgM+ B cells.

However, antigen-related terminal differentiation and expansion of IgM and IgA B cells appear to be normal, as does the trans-epithelial immunoglobulin transport (Baklien 1977). The finding of a similar proportion of IgA bound to SC in jejunal fluid samples from CIA positive and CIA negative patients (see Chapter V) does not support the hypothesis that an abnormally high capacity for transport of IgM and IgA across the epithelium might produce the CIA pattern. If the affinity for the SC for IgM is much higher than for dimeric IgA, then over-production of IgM in the lamina propria may partially block trans-epithelial IgA transport, which gives an explanation for the presence of dimeric IgA in the serum in coeliac disease (Troncone 1991b).

The higher number of IgM+ plasma cells found in non-coeliac CIA positive patients as compared to CIA negative, is evidence of an apparent imbalance of lamina propria plasma cells in the first group of patients. These results confirm data shown in Chapter VI, validate the finding of the CIA pattern, and support the view that this antibody pattern is locally produced. Moreover, this finding, together with the high proportion of jejunal fluid sIgA found in the CIA positive group, suggests that neither the B cell population, nor the transepithelial

immunoglobulin transport, are affected, but rather that it is the immuno-regulatory factors that control these processes.

The lack of correlation between lamina propria IgM+ plasma cell counts and IgM concentrations may suggest that they are unrelated. CIA positive patients have a high concentration of IgM and a polyclonal IgM antibody response. The intra-cellular form of IgM (mainly monomeric) detected by immuno-histochemical studies may be different to the secreted form (polymeric) detected in jejunal fluid by ELISA, as occurs for IgA (Moldoveanu 1984). It is also possible that in CIA positive patients, the secreted low-affinity and polyclonal IgM molecule is rapidly cleaved or is not functional, and its levels are not paralleled by the number of specific plasma cells at a given time. Only a small proportion of the IgA and IgM-producing plasma cells are involved in anti-gliadin antibody production, as shown by in vitro culture of untreated biopsies (Ciclitira 1986b, Lycke 1989).

The number of IgM+ B cells may also be secondary to gluten ingestion in sensitized individuals, and as in other mucosal changes observed in coeliac patients, adults maintain these abnormalities even after long periods of GFD, as compared with children who have a higher potential for mucosal normalization (Baklien

1977, Scott 1980a, Savilahti 1972).

CHAPTER IX

OTHER STUDIES IN NON-COELIAC PATIENTS

Introduction

Markers of potential coeliac disease may be integrated in the spectrum of gluten-sensitivity in two ways, representing either the primary mucosal abnormality (see Chapter VIII); or as subtle evidence of the existence of an ongoing hypersensitivity reaction activated either by a local immuno-stimulating factor or by other mechanisms. I pursued the latter hypothesis and carried out various studies, described in this chapter.

Within the spectrum of expression of a low-grade mucosal DTH reaction, the CIA pattern may be positive even before the IEL count is raised. Intestinal immune activation is manifested by IEL infiltration and HLA-DR expression by crypt epithelium (Marsh 1980, Leigh 1985). Within the lamina propria there is a polyclonal activation of CD4+ cells and expression of the CD25 marker (Griffiths 1988, Haltensen & Brandtzaeg 1993); local production of prostaglandins and soluble factors (Lavo 1990, Branski

1992); increased number of plasma cells (Scott 1980a, Dhesi 1984); infiltration of basophils, neutrophils, eosinophils, MMC and goblet cells (Dhesi 1984, Marsh & Hinde 1985), evidence of cellular activation and degranulation (Hallgren 1989, Norvath 1989, Colombel 1992, Desreumaux 1992, Scott 1992) and complement activation (Haltensen 1992a).

Cultured coeliac biopsies release cytokines into the supernatant after gluten challenge (Ferguson 1975). These products may mediate morphological and functional changes induced by gluten-specific T cells. It is known that gluten challenge induces a dose-dependent IEL infiltration, followed by activation of lamina propria cells (Marsh 1980, Leigh 1985, Monk 1988) and expression of HLA-DR antigens predominantly by crypt epithelium (Ciclitira 1986a). Activated IELs produce IFN γ which modulates the epithelial HLA-DR expression (Scott 1981, Cerf-Bensussan 1984), and this may be related to the induction of a secondary inflammatory reaction (Mayer 1991). Expression of HLA-DQ molecules on the lamina propria may also be involved in antigen presentation. There is an exciting new report where gluten-specific cells, characterized as HLA-DQ restricted T cells, have been cloned (Lundin 1993).

It was thought that patients positive for markers of

potential coeliac disease, eg CIA positive, high total IEL and $\gamma\delta$ + IEL counts, might have an active low-grade DTH reaction in an otherwise morphologically normal mucosa. This might be manifested by the presence of high numbers of MMC in lamina propria, HLA-class II antigen expression by jejunal epithelial cells (particularly HLA-DR in crypt epithelium), and/or by the cell activation marker CD25 (which identifies the IL-2R α chain). Eventually, I wanted to establish whether the existence of these markers correlated with the expression of other indices of potential coeliac disease.

During the study to characterize jejunal fluids as material for the study of mucosal immunity, a colleague (Dr Obedy Mwantembe) found detectable levels of soluble IL-2R and TNF α in whole gut lavage fluid samples. This finding encouraged me to measure these cytokines in jejunal fluid specimens using the same tests. In addition, an ELISA test for IFN- γ was developed in the laboratory (by Louise Handy), and this was added to the battery of cytokine assays performed in jejunal fluids.

I also studied a group of treated coeliac patients. This group was subdivided either by dietary status or by mucosal histology when compared with the group of non-coeliac patients, to evaluate differences in cytokine levels.

A. Evidence of "DTH" effect on crypt epithelial cells

Though the study was carried out to assess the expression in general of HLA-DR and DQ antigens by the intestinal mucosa, I was looking in particular at the significance of the IEL population in the epithelial compartment. Demographic data of patients studied are shown in Table IX.1. Coeliac patients were grouped according to biopsy histology and dietary status; and non-coeliac patients were categorized by the total IEL count and the expression of the CIA pattern.

HLA-DR antigen expression was observed in the villus epithelium of almost all specimens studied, showing a general staining pattern with maximum expression at the tip of the villi affecting the basolateral membrane of enterocytes, and decreasing towards the crypt epithelium, where the staining was almost absent. This pattern was particularly expressed by untreated coeliacs with PVA or STVA, where positivity for HLA-DR in crypt epithelium and the presence of lamina propria CD25+ cells seem to be the most specific markers of T cell activation.

No differences were found in the HLA-DR expression by the villous epithelium when coeliac patients with a morphologically normal biopsy and non-coeliac patients

TABLE IX.1. DEMOGRAPHIC DATA OF PATIENTS STUDIED FOR THE EXPRESSION OF HLA-CLASS II ANTIGENS AND THE CD25 MARKER IN JEJUNAL BIOPSIES.

DIAGNOSIS	(n)	AGE median (range)	SEX f/m	DIET NORMAL GFD	HISTOLOGY NORMAL MINOR PTVA CHANGES
COELIAC					
Untreated	7	28.5 (12-54)	4/3	7	7
Challenge	1	15	1/-	1	1
Treated	8	46 (21-62)	6/2	8	3 5
NOT COE	1	30	-/1		1
POT COE	1	48	1/-	1	1
IBD	1	45	1/-	1	1
DIA IDIO	4	29 (24-36)	2/2	4	1 3
ORAL ULC	3	30 (28-30)	2/1	3	1 2
MISC	4	57.5 (43-72)	3/1	4	1 3
NUT DEF	3	35 (29-43)	3/-	3	1 2
IBS	10	30 (20-45)	7/3	10	8 2
NAD	5	36 (20-59)	4/1	5	3 2

NOT-COE= previous diagnosis on inadequate criteria

POT COE= potential coeliacs on follow up studies

IBD= inflammatory bowel disease

IDIO DIA= idiopathic diarrhoea

ORAL ULC= recurrent mouth ulcers

MISC= significant organic GI disorders

NUT DEF= nutritional deficiencies due to poor diet

IBS= irritable bowel syndrome

NAD= trivial symptoms, psychiatric disease

GFD= gluten-free diet

Minor Changes= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

with or without high IEL counts were compared (Table IX.2). The intensity of staining was, in general, higher in the villous epithelium than in the crypts.

In crypt epithelium, 14 of the 16 coeliac patients were positive, as were 12 of the 16 non-coeliac cases with high total IEL counts (frequencies not statistically different). By contrast, only 3 of the 16 non-coeliac cases with normal IEL counts had positive HLA-DR staining in crypt epithelium, and the frequency was significantly lower when compared to coeliacs ($p < 0.001$) and non-coeliac patients with high IEL counts ($p < 0.01$) (Table 23).

When coeliacs were subdivided by diet, no differences were found between either the group on a normal diet or on a GFD or non-coeliac patients with high IEL counts. However, both coeliac groups had higher HLA-DR expression in crypt epithelium ($p < 0.01$) when compared with non-coeliacs with normal IEL counts. On the other hand, only non-coeliac CIA negative patients had a significantly lower HLA-DR expression in the crypt epithelium when compared to coeliac patients either as a whole ($p < 0.001$) or on a normal diet ($p < 0.05$).

HLA-DQ expression was absent in villus and crypt epithelium from all the sections. However, positive cells

TABLE IX.2. POSITIVE EXPRESSION OF HLA-CLASS II ANTIGENS IN JEJUNAL BIOPSIES FROM COELIAC AND NON-COELIAC PATIENTS

		HISTOLOGY			HLA-DR			HLA-DQ			CD25+
(n)		PTVA	MIN	N	VE	CE	LP	VE	CE	LP	
COELIAC											
I. NORMAL											
DIET: CIA+		(7)	7		7	7	7			7	7
CIA-		(2)	2		2	1	2			2	2
II.GFD: CIA+											
CIA+		(5)	4	1	5	5	5			4	3
CIA-		(2)	1	1	2	1	2			1	1
NON-COELIAC											
I. HIGH											
IEL: CIA+		(8)	8		8	7	8			8	1
CIA-		(8)	8		8	5	8			8	1
II.NORMAL											
IEL: CIA+		(4)		4	4		4			4	
CIA-		(12)		12	10	3	12			12	

PTVA=severe partial or total villus atrophy
MIN CHAN=high total IEL counts
VE=villous epithelium
CE=crypt epithelium
LP=lamina propria

with an heterogeneous morphology, were found in the lamina propria of most specimens. No significant differences were found when groups were compared. Positive cells may be numerically similar between coeliac and non-coeliac groups: however, the staining pattern is stronger in the coeliac group, and in some of these cases these cells had a subepithelial localization (Table IX.2).

B. Evidence of lamina propria cell activation

Counts of lamina propria MMC were performed in biopsy sections from the same group of patients studied for lamina propria plasma cell counts (see Chapter VIII). A description of patients is shown in Table VIII.1. Mucosal mast cell counts were not significantly different when both treated and untreated coeliac patients were compared to non-coeliac patients either positive or negative for the expression of the CIA pattern (Figure IX.1).

Studies of expression of the CD25 cell marker were performed in the same cases as for HLA-DR and DQ expression and they are shown in Table 7 (see Chapter V). Positive cells for the CD25 marker were only found in the lamina propria of coeliac patients in 13 of the 16 cases studied, and this was particularly related to the co-existence of PVA or STVA, but not with the dietary

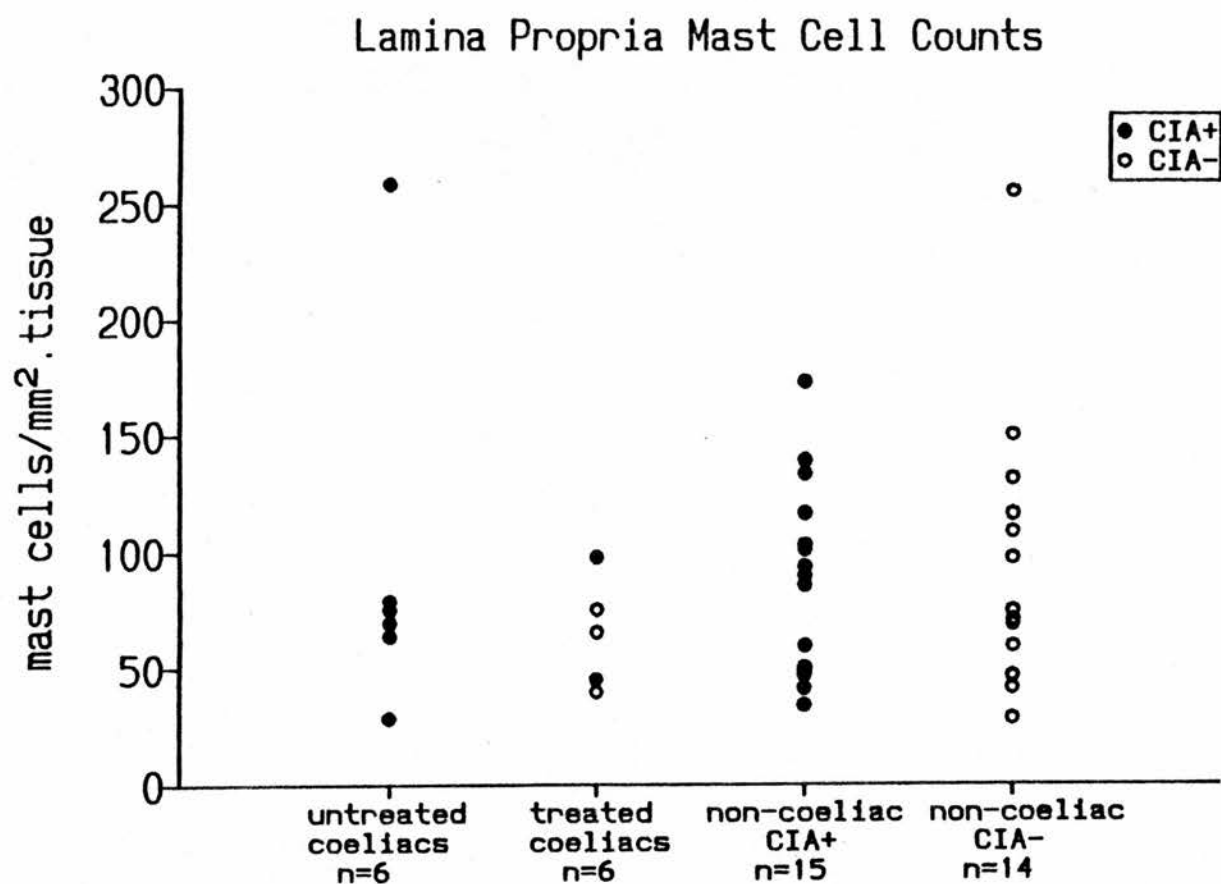


Figure IX.1: Lamina propria MMC counts per square mm of tissue in treated (n=5) and untreated (n=7) coeliacs, and non-coeliac patients positive (n=15) and negative (n=14) for the CIA pattern.

status. When compared with non-coeliac patients, coeliacs had a higher lamina propria CD25+ cell expression ($p < 0.001$).

Amongst non-coeliacs, only two patients had any lamina propria CD25+ cells: a woman with recurrent mouth ulcers and positive for all three markers of potential coeliac disease, who later developed a gluten-sensitive enteropathy (see Chapter X); and a man with idiopathic diarrhoea, high total IEL and $\gamma\delta$ IEL counts, but CIA negative. Both also had positive HLA-DR expression in crypt epithelium.

Cytokines in jejunal fluid samples

I. Measurement of soluble IL-2R levels.

Jejunal fluid samples were studied from patients described in Table II.4. Results of soluble IL-2R levels are shown in Figure IX.2. Mean values and comparison between groups are also shown in Table IX.3. The upper reference range for IL-2R (138 IU/ml) was calculated using the 95% confidence interval of the values obtained by the mainly normal or control group. Samples had high soluble IL-2R levels when above this value. The control group includes patients with a diagnosis of a functional bowel disorder, or in whom no abnormality was detected

The figure consists of two vertically aligned scatter plots sharing a common x-axis. The top plot's y-axis ranges from 0 to 1200 with increments of 200. The bottom plot's y-axis ranges from 0 to 250 with increments of 50. A break in the y-axis is indicated by two parallel diagonal lines between the two plots. The legend in the top plot indicates that open circles represent 'NORMAL' and filled circles represent 'PTVA'.

Top Plot Data (Estimated):

Group	IgA Level
NORMAL	500
NORMAL	1010
PTVA	410

Bottom Plot Data (Estimated):

Group	IgA Level
CONTROL GROUP	0
CONTROL GROUP	20
CONTROL GROUP	25
CONTROL GROUP	30
CONTROL GROUP	40
CONTROL GROUP	50
CONTROL GROUP	60
CONTROL GROUP	70
CONTROL GROUP	105
CONTROL GROUP	180
CONTROL GROUP	215
COELIACS NORMAL DIET	10
COELIACS NORMAL DIET	30
COELIACS NORMAL DIET	35
COELIACS NORMAL DIET	40
COELIACS NORMAL DIET	55
COELIACS NORMAL DIET	100
COELIACS ON GFD	5
COELIACS ON GFD	10
COELIACS ON GFD	15
COELIACS ON GFD	20
COELIACS ON GFD	25
COELIACS ON GFD	30
COELIACS ON GFD	50
COELIACS ON GFD	70
COELIACS ON GFD	90
COELIACS ON GFD	95
COELIACS ON GFD	100
COELIACS ON GFD	120
COELIACS ON GFD	145
COELIACS ON GFD	165
COELIACS ON GFD	245
ORGANIC DISORDERS	0
ORGANIC DISORDERS	10
ORGANIC DISORDERS	15
ORGANIC DISORDERS	20
ORGANIC DISORDERS	25
ORGANIC DISORDERS	30
ORGANIC DISORDERS	35
ORGANIC DISORDERS	40
ORGANIC DISORDERS	45
ORGANIC DISORDERS	50
ORGANIC DISORDERS	60
ORGANIC DISORDERS	70
ORGANIC DISORDERS	80
ORGANIC DISORDERS	90
ORGANIC DISORDERS	115
ORGANIC DISORDERS	135
ORGANIC DISORDERS	140
ORGANIC DISORDERS	150

Normal= morphologically normal mucosa, PTVA= severe partial or total villus atrophy

TABLE IX.3. SOLUBLE IL-2R AND TNF α LEVELS IN JEJUNAL FLUID

	(n)	IL-2R LEVELS (UI/ml)	TNF α LEVELS (pg/ml)
CONTROL	28	29.7 , 54 (0-215)	7.4 , 16.5 (0-70)
ORGANIC DISEASE GROUP	28	*96.7 , 197 (0-1011)	12.6 , 21 (0-75)
COELIAC GROUP			
A.BY MUCOSAL APPEARANCES			
NORMAL	8	*86.2 , 83 (0-245)	9.5 , 12.6 (0-32)
PTVA	16	*78 , 97.5 (3-404)	+24.7 , 31.6 (0-125)
B.BY DIET:			
NORMAL	5	45.6 , 33.5 (9-98)	+26.8 , 17.6 (0-49)
GFD	19	*151.3 , 314 (0-404)	+16.5 , 28.4 (0-125)

PTVA= severe partial, or total villus atrophy

GFD= gluten-free diet

* Patients with organic disorders, and coeliacs with either normal and PTVA ($p<0.05$) or on GFD ($p<0.001$) had higher levels of soluble IL-2R than controls.

+ Coeliac patients either with PTVA or on normal and gluten-free diets had higher levels of TNF α ($p<0.05$) than controls.

Data expressed as the mean, standard deviation (range values)

after investigation.

In the control group, two of the 28 patients had high IL-2R levels (Table IX.3), a woman with a positive CIA and a high total IEL count; and a second case with a high $\tau\delta$ IEL count, but no other abnormalities, who was finally diagnosed as having a functional bowel disorder. Control group patients had significantly lower IL-2R levels when compared with both non-coeliac patients with a significant GI disease ($p<0.05$), and coeliac patients whether treated or untreated ($p<0.05$).

Coeliac patients were subdivided using the dietary status and the jejunal biopsy histology. High soluble IL-2R levels were found in 3 of the 8 coeliac patients with normal histology, and 2 of the 16 patients with either PVA or STVA. Both treated and untreated coeliac patients had similar IL-2R levels when compared to non-coeliac patients with other organic GI diseases (Table IX.3).

Three of the patients with other organic GI disorders also had high levels of soluble IL-2R, a man with diarrhoea due to high alcohol intake, and two women, one with a diagnosis of collagenous colitis, the second with idiopathic diarrhoea, and positive for the 3 markers of potential coeliac disease. Positive control cases and non-coeliac patients with other organic diseases are

described in Table IX.4.

II. Measurement of TNF α levels.

Jejunal fluid samples were studied in the same cases as for soluble IL-2R (see Table II.4). Results of TNF α levels are shown in Figure IX.3. Mean values and comparison between groups are also shown in Table IX.3. The upper reference range for TNF α (40 pg/ml) was calculated using the 95% confidence interval of the values obtained from the control group. Samples had high TNF α levels when above this value. One of the patients from the control group had high TNF α levels, this was a woman in whom no diagnosis was reached after full investigation.

Three coeliac patients with an atrophic-villous mucosa, but none of the cases with a normal histology had high TNF α levels. Compared to the control group, coeliac patients with PVA or STVA had higher TNF α levels ($p < 0.05$), and these levels were also higher than those for non-coeliac patients with an organic GI disease and normal biopsy histology, though this difference was not statistically significant (Table IX.3).

TNF α levels were high in 4 of the patients with organic GI disorders, two men with a diagnosis of pancreatic

TABLE IX.4. DESCRIPTION OF NON-COELIAC CASES WITH HIGH LEVELS OF SOLUBLE IL-2R IN JEJUNAL FLUID

DIAGNOSIS	CIA PATTERN	HIGH TOTAL IEL COUNT	HIGH $\tau\delta+$ IEL COUNT	COMMENT
NAD	+	+	-	
IBS	-	-	+	
IDIO DIA	+	+	+	enteropathy after challenge
EXCESS ALCOHOL	-	-	-	
COLLAGENOUS COLITIS	-	-	-	Trial of GFD

[illegible]

Normal= morphologically normal mucosa, PTVA= severe partial or total villus atrophy

cancer and recurrent mouth ulcers, respectively, but no other abnormalities; and two women, both with idiopathic diarrhoea, one the case positive for 3 markers of potential coeliac disease and high levels of soluble IL-2R in jejunal fluid. A description of the control case and the 4 non-coeliac patients with high $\text{TNF}\alpha$ levels are shown in Table IX.5.

III. Measurement of $\text{IFN}\gamma$ levels.

Suitable jejunal fluid to measure $\text{IFN}\gamma$ levels was available from 37 of the 82 patients studied for soluble IL-2R and $\text{TNF}\alpha$ (see Chapter II). Results of $\text{IFN}\gamma$ are shown in Figure IX.4, and reference values obtained by the study groups are given in Table IX.6. The sensitivity of the ELISA test for jejunal fluid $\text{IFN}\gamma$ was >4 IU/ml. None of the control group patients had detectable levels above this value.

Two coeliac patients: one treated and one on a normal diet (both with P/TVA), and two DH patients, one treated and one on a normal diet (both with minimal histological changes in their biopsies), had detectable $\text{IFN}\gamma$ levels in jejunal fluid.

Four of 15 patients with organic GI disorders also had detectable $\text{IFN}\gamma$ levels: three women, one with idiopathic

TABLE IX.5. DESCRIPTION OF NON-COELIAC CASES WITH HIGH LEVELS OF TNF α IN JEJUNAL FLUID

DIAGNOSIS	CIA PATTERN	HIGH TOTAL IEL COUNT	HIGH $\tau\delta+$ IEL COUNT	COMMENT
NAD	-	-	not done	
IDIO DIA	+	+	+	enteropathy after challenge
IDIO DIA	-	-	-	
PANCREATIC CARCINOMA	-	-	not done	
ORAL ULCERS	-	-	not done	

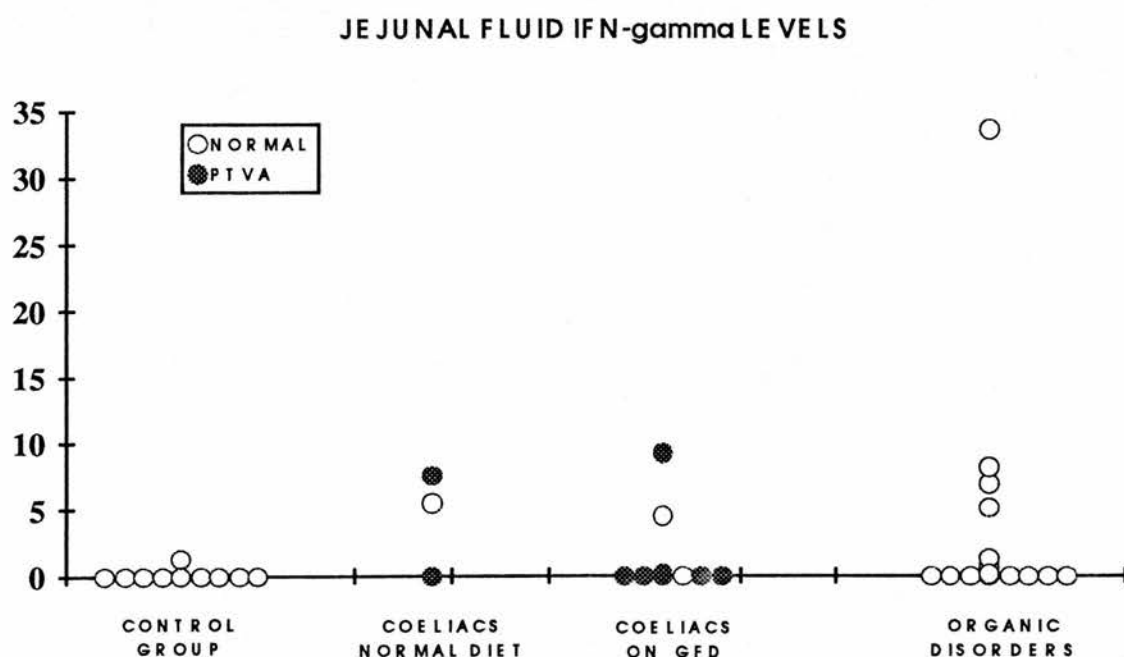


Figure IX.4: Jejunal fluid IFN γ levels expressed as IU/ml, in coeliac/DH patients (n=12), non-coeliac patients with organic GI disorders (n=15), and controls (n=10).

Normal= morphologically normal mucosa, PTVA= severe partial or total villus atrophy

TABLE IX.6. LEVELS OF IFN γ LEVELS IN JEJUNAL FLUID

	NUMBER OF CASES	IL-2R LEVELS (UI/ml)
CONTROL GROUP"	10	0.13 , 0.4 (0-0.13)
ORGANIC DISEASE GROUP	15	3.7 , 8.7 (0-33.7)
COELIAC/DH GROUP		
A.BY MUCOSAL APPEARANCES:		
NORMAL	5	2 , 2.7 (0-5.3)
PTVA	7	2.4 , 4 (0-9.2)
B.BY DIET:		
NORMAL	3	4.3 , 3.9 (0-7.6)
GFD	9	1.5 , 3.2 (0-9.2)

PTVA=severe partial, or total villus atrophy

Data expressed as the mean, standard deviation (range values)

diarrhoea who was CIA positive and had a high total IEL count; and two with recurrent mouth ulcers, one of whom was CIA positive and had a high total IEL count; as well as a man with a diagnosis of ulcerative colitis. Descriptions of positive cases are shown in Table IX.7.

Differences between groups were not significant: the group of untreated coeliacs was very small, however. No positive correlations were found when cytokine levels were compared within the group of 37 patients in whom samples were available for measurements of soluble IL-2R, $\text{TNF}\alpha$, and $\text{IFN}\gamma$.

Conclusions and implications of these results

Evidence of mucosal T cell activation can be found in the intestine of non-coeliac patients positive for some markers of potential coeliac disease, without any major change in villous architecture. These include polyclonal expansion of cells in mucosa (eg IgM+ plasma cells), production of cytokines, and expression of cell activation markers in jejunum. This suggests the existence of an inflammatory process in the intestine. HLA-DR expression by crypt epithelium is more frequent amongst patients with high IEL counts; whereas lamina propria CD25+ cells are only found in patients with villous atrophy, or minor histological abnormalities.

TABLE IX.7. DESCRIPTION OF NON-COELIAC CASES WITH HIGH LEVELS OF IFN γ IN JEJUNAL FLUID

DIAGNOSIS	CIA PATTERN	HIGH TOTAL IEL COUNT	HIGH $\gamma\delta$ + IEL COUNT	COMMENT
UC	+	-	not done	
IDIO DIA	+	-	not done	
ORAL ULCERS	-	-	not done	
ORAL ULCERS	-	-	not done	

Similar results have been obtained in a proportion of healthy first degree coeliac relatives with a normal biopsy, and HLA-DR expression was found to be correlated with the coeliac-associated DQ gene markers. These patients are potential coeliacs with high $\gamma\delta$ + IEL counts, and may be monitored for the development of histological changes by screening for positive (seroconversion) ARA and AEm antibodies (Holm 1993b). In coeliac patients, activation markers (HLA-DR, CD25) are expressed in parallel with the $\alpha\beta$ + cell infiltration of the epithelium (not by $\gamma\delta$ + cells), which is related to the stage of the disease (Kutlu 1993).

The coeliac lesion is due to an aberrant mucosal immune response to gluten that seems to be mediated by HLA-class II restricted lamina propria cells (O'Farrelly & Gallagher 1992). MHC products may determine the immune responsiveness to gluten, whether by repertoire selection in thymus or by antigen presentation at local level, by HLA-DR+ enterocytes or more likely by HLA-DQ+ antigen-presenting cells. HLA molecules do not bind gliadin as lectins, and there is no cross-reactivity with gliadin (Gallagher 1988). However, gluten specific HLA-DQ restricted T cells have been isolated from jejunal coeliac biopsies (Lundin 1990, 1993).

Experiments of polyclonal T cell activation of human

mucosa in vitro induce crypt cell hyperplasia, expression of CD25 markers by T cells and macrophages, lymphokine release, IEL infiltration, and increased HLA-DR expression by enterocytes and crypt epithelial cells (MacDonald & Spencer 1988, MacDonald 1990; Fais 1992). These features are also found in untreated coeliacs, with lamina propria expressing the CD25+ marker, whereas the epithelium expresses the proliferative antigen identified by the mAb Ki-67 (Haltensen & Brandtzaeg 1993). This suggests that IELs are induced to proliferate independently of the antigen (thus the absence of gluten-specific T cells), but probably dependent on IL-2 production secondary to lamina propria CD4+ cell activation.

In agreement with others, these results show that villous epithelial cells normally express HLA-DR antigens. However, HLA-DR expression by crypt epithelium is more frequently associated with a high IEL count, without any other histological change. The frequency of positive cases was similar in coeliacs and non-coeliacs with high IEL counts, though the intensity of staining was stronger in the first group, suggesting that it may represent an early inflammatory change in non-coeliacs. CIA positive patients had a similar frequency of cases with positive cells in crypt epithelium to coeliacs. HLA-DQ antigens are less numerous, but positive cells are found in lamina

propria, some of them big, macrophage-like cells.

I am aware that the jejunal fluid samples I have used to measure cytokine levels had been previously thawed and frozen at least once (for total immunoglobulins and specific antibodies levels), therefore the results have to be evaluated with caution. However, in order to give a complete report of the work carried out on these patients, cytokine data has been included. It is clear that if a definitive study on cytokine levels in jejunal secretions is to be compiled it will be necessary to use "fresh" aliquoted jejunal fluid samples.

The lack of correlation between jejunal fluid IL-2R and TNF α levels and markers of potential coeliac disease may be due to the short lifetime and rapid clearance of cytokines from fluids. As the reliability of the assay depends on rapid treatment and storage of samples, the samples in this study were not ideal. Independently of the mucosal status, non-coeliac patients with organic GI disorders had similar IL-2R levels to coeliacs, whereas only the latter have high TNF α levels in association with the presence of an atrophic-villous mucosa. IFN γ was found to be positive only in patients with organic disease, including one who later developed a gluten-sensitive enteropathy and was positive for the two other cytokines and 3 markers of potential coeliac disease.

IFN γ modulates the epithelial function and expression of surface markers HLA-DR and SC (Cerf-Bensussan 1984, Madara & Stafford 1989, Scott 1987, Sollid 1987). In coeliac disease, IFN γ -secreting cells are increased in relation to the degree of villous atrophy (Kontakou 1993). Serum soluble IL-2R levels may be an indirect index of immune activation by dietary gluten in coeliacs, perhaps related to CD4+ cell stimulation (Crabtree 1986b, Blanco 1992). Lamina propria TNF α producing cells and macrophages are also increased in active coeliac disease (Przemioslo 1993). However, TNF α has a short life and, though it may be produced by activated macrophages and Paneth cells it is normally undetectable in secretions. A great proportion of IELs, with memory phenotype, may synthesize IL-2, IL-5, and/or IFN γ .

Further elucidation of the relationships between the immunological phenomena described and the clinical entity of coeliac disease will require extension of the studies described in this chapter, together with direct, "in-vivo" investigations of gluten sensitivity in potential coeliac patients, as well as characterization of the genetic make-up of both coeliac and non-coeliac patients, as to whether they do or do not have the various immune abnormalities described above.

CHAPTER X

CLINICAL EFFECTS OF GLUTEN-FREE DIET AND GLUTEN LOADING

Introduction

One of the implications of the study of the expression of candidate markers of latent coeliac disease in symptomatic non-coeliac patients, some of them found to be positive for one or more, is the need for a complete re-think of the basis for selecting patients who need to undergo a GFD trial, and consequently the revision of diagnostic criteria of the disease. There has been a previous report of gluten-sensitive diarrhoea, describing benefit from a GFD in 9 of 17 patients suffering from chronic diarrhoea, but their jejunal biopsies were not normal (Cooper 1980).

In this laboratory, Kathleen Kingstone is currently working on the characterization of coeliac and non-coeliac patients using genetic markers associated with the HLA-DQ locus, and their relationship to different patterns of expression of candidate markers of latent coeliac disease. Patients positive for at least 2

indices, who may express the HLA-DQ α/β heterodimer, should be followed up. However, the diagnosis of latent coeliac disease may be confirmed earlier by a clinical trial of gluten sensitivity, and if confirmed, a GFD can be recommended in these patients.

Although clinical effects of treatment are important to the patient, objective measures of the effects of GFD in coeliac disease are mandatory for clinicians and investigators. Thus histopathological improvement currently forms part of the definition of the disease. Objective assessment of a response to a GFD in the present series of patients will only be possible in patients who have a functional disorder (eg abnormal permeability), or those with minor biopsy abnormalities within the classical spectrum of enteropathy (eg high IEL counts). This is independent of the remission of symptoms.

Trials of gluten-free diet in non-coeliac patients

This study was carried out in an opportunistic, non systematic way, selecting patients on the basis of the presence of symptoms or the finding of a high total IEL count. The 14 patients studied were offered a clinical trial of gluten restriction. These were non-coeliac non-DH patients, 8 were CIA positive at the time of the first

biopsy on a gluten-containing diet, and 6 were CIA negative. Details are presented in Table X.1: 8 cases had high IEL counts (5 CIA positive), one had villus shortening and a normal IEL count, and 5 had a morphologically normal biopsy. At the time of completion of this thesis (03.12.93) two of these patients are on the list for a second biopsy.

Five CIA positive and 2 CIA negative patients with chronic high-volume watery diarrhoea had complete and rapid resolution of diarrhoea within 1-2 weeks of starting a gluten-free (or, in one case, low gluten) diet; in four of the CIA positive cases, the IEL count in the initial biopsy was high, and in the three cases in which repeat biopsy has been performed, the IEL count has fallen to normal. There are two CIA negative patients (JS, AS) who showed a good clinical response, and a reduction in the IEL count (though in the first one, the count was still high).

After 6 months of GFD a partial improvement was observed in the severe watery diarrhoea of a woman with collagenous colitis (CS) whose identical twin is a coeliac. She was CIA negative, her jejunal biopsy was morphologically normal with an IEL count of 31% and no $\gamma\delta$ + IELs. She is currently on a GFD. The trial of GFD had no effect in a CIA positive patient (CR), with ulcerative

colitis and sclerosing cholangitis (on 10 mg/day of prednisolone), who had a high-volume watery diarrhoea after colectomy and formation of a pelvic ileal pouch.

Development of a mucosal lesion in a case of potential coeliac disease after gluten loading

There were three patients of particular interest, positive for 3 markers of potential coeliac disease. One of these patients defaulted from follow-up. The other two have been shown to be gluten-sensitive (see above). One of the cases (PK) had resolved her chronic high volume diarrhoea within 2 weeks of starting the trial, and IEL count was normal in a biopsy taken 2 months later.

The second case (JP) was a 41 year old, CIA positive woman with recurrent severe oral ulceration when eating a diet with 24 g gluten daily, despite a reportedly morphologically normal jejunal biopsy, but the presence of an abnormal permeability test. She was prescribed a GFD and she noticed some improvement but found the diet inconvenient and kept to it for only five weeks. Several months later, when studies of TCR $\gamma\delta$ + IEL in a stored biopsy specimen were performed and counts were found to be high, she agreed to take 10 g additional gluten daily for a month and had another jejunal biopsy. Pathological examination revealed STVA (classical coeliac-like

enteropathy). This patient was one of the two non-coeliac cases with CD25+ cells in jejunum.

Gluten sensitivity and potential coeliac disease

Around 40% of patients referred for diagnostic jejunal biopsy, and in whom routine biopsy pathology was normal, had positive results for one or more of the tests that have been proposed as indices of latent coeliac disease. Although evidence of clinical and mucosal gluten sensitivity has been obtained in a few of these patients (Arranz 1993), this was done on an opportunistic basis, unsystematic and uncontrolled, and further careful studies are now essential.

Of the 31 patients with a positive result for one or more indices of potential coeliac disease, there were 19 with a single abnormal test. Of particular interest are the three patients with 3 parameters positive: CIA pattern, $\gamma\delta$ + IEL count and total IEL count. Two of the three, both women, were shown to be gluten-sensitive after a trial of GFD; and one of them (JP) was the patient who underwent a trial of gluten loading, and developed a gluten-sensitive enteropathy (see above).

Induction of severe enteropathy by extra dietary gluten would be unequivocal proof of gluten sensitivity, but

this raises ethical issues and may be clinically unacceptable to these patients. Acute enteral or rectal gluten challenge, monitored by multiple biopsies, should be practicable. However, since the histopathological effects of dietary gluten in clinically gluten-sensitive patients with normal jejunal biopsy are strikingly different from those in classical coeliacs, it is entirely possible that the pathological changes produced by acute gluten challenge in the jejunum or rectum will be different in latent or potential coeliacs as compared with classical coeliacs, so a range of pathological and molecular indices will still require measurement in future work.

Clinical details of patients and investigations

(Note: when biopsy is referred to, this is a jejunal biopsy unless otherwise stated.)

Mrs CR. A 24-year-old woman diagnosed as having ulcerative colitis and sclerosing cholangitis, on 10 mg of prednisolone daily. She developed a high-volume watery diarrhoea after colectomy and formation of a pelvic ileal pouch. Her first biopsy showed minor villus shortening and a total IEL count of 15%, she had a CIA positive pattern in her jejunal fluid, and the sugar permeability test was grossly abnormal (0.240). Serum anti-gliadin

antibodies were negative. No effect was found after 6 months on a GFD, her second biopsy showed similar histological changes (IEL count of 16%), and the sugar test was abnormal (0.104).

Mrs DM. This 19-year-old woman was investigated because of a 6 month history of watery diarrhoea with some weight loss. Her daily gluten intake was 37 g. Her first jejunal biopsy was morphologically normal, but the total IEL count was very high (80%). The diagnosis was made of idiopathic diarrhoea. She had a CIA positive jejunal fluid, high levels of IgG anti-gliadin antibodies in serum, and the sugar permeability test was normal (0.012). Her diarrhoea resolved within 2 weeks of starting a low gluten diet (5 g daily), and a reduction of the IEL count to 23% was observed in her second jejunal biopsy. She has been advised to maintained an strict GFD indefinitely.

Mrs DP. This 54-year-old woman presented with watery diarrhoea. She was investigated by a colonoscopy, and a colonic biopsy taken showed microscopic colitis. She had no response to trials of prednisolone and sulphasalazine. The study of her jejunal biopsy and fluid also revealed a morphologically normal mucosa with a high IEL count (46%), and a CIA positive pattern, respectively. The serum AGA test was negative, and the sugar permeability

test was within the normal range (0.032). Her diarrhoea resolved after one week of GFD. The second biopsy showed an IEL count of 36%. She is currently on a GFD.

Mrs PK. A 23-year-old woman investigated by jejunal biopsy because of chronic diarrhoea and fatigue. Her jejunal biopsy had a normal villous architecture but the IEL count was increased (61%), with an high proportion of epithelial $\tau\delta$ + cells (80/mm). Only the serum IgG anti-gliadin antibody titer was slightly high, but IgA ARA/AEm antibodies and the jejunal fluid CIA pattern, were positive. The sugar permeability test was normal (0.020). She had a good clinical response to a GFD, with remission of diarrhoea within two weeks. Her second biopsy showed a reduction in the IEL count to 38%.

Mrs KW. This 26-year-old woman presented with intermittent watery diarrhoea, colicky abdominal pain, lethargy, and iron deficiency anaemia. Her jejunal biopsy showed a normal villous architecture but a high IEL count of 44%, and an increased chronic inflammatory cell infiltrate in the lamina propria. Serum antigliadin antibodies were not detected, and the sugar permeability test was abnormal (0.065). She had a CIA positive jejunal fluid. Diarrhoea resolved within a week of starting a GFD, but she defaulted from further appointments.

Mrs HS. This 52-year-old woman was investigated by jejunal biopsy because of iron deficiency anaemia and idiopathic diarrhoea. Her biopsy was normal with an IEL count of 21%. She had a CIA positive jejunal fluid, serum anti-gliadin antibodies were negative, and an abnormal sugar permeability test (0.062). Her second biopsy was normal with a total IEL count of 23%, but the sugar test was still abnormal (0.074). She is currently on a GFD.

Mrs JP. This 41-year-old woman had a history of recurrent severe mouth ulcers related to her menstrual period. Her diet contained 24 g of gluten daily. A diagnosis of iron deficiency anaemia was made in 1989, but she had irrelevant symptoms or family history. Her first jejunal biopsy was morphologically normal, with an IEL count of 50% and a high number of $\gamma\delta$ + cells (51 cells per mm of epithelium). The sugar permeability test was abnormal (0.079), she had 12 g/dl of haemoglobin and a low level of ferritin (<5 ug/ml). She had a CIA positive jejunal fluid, and high titers of serum IgG and IgA anti-gliadin and IgA ARA/AEm antibodies. Her mouth ulcers improved clinically after 2-3 weeks on a GFD, but she decided to recommence a normal diet, and a trial of gluten loading was then performed. The jejunal biopsy taken at that time showed STVA, but no changes in her mouth ulcers. The symptoms disappeared after gluten restriction, with iron and folate supplements. She is currently being treated

with a GFD and has been recommended to remain on this diet indefinitely.

Mrs HS. This 45-year-old woman was previously diagnosed as having Crohn's disease, which has been clinically inactive. However, she developed a severe diarrhoea after undergoing a limited ileal resection for active disease, and the diarrhoea was unresponsive to Cholestyramine. Her first jejunal biopsy was normal with an IEL count of 36%, but a high epithelial $\gamma\delta$ + cell count (8.2 cells per mm) was found in frozen sections. She had a CIA positive jejunal fluid, serum anti-gliadin antibodies were not detected, and the sugar permeability test was abnormal (0.052). There was no clinical effect after two months of a GFD, no further investigations were done, and she returned to a normal gluten-containing diet.

Mrs CS. This is a 68-year-old woman with a diagnosis of collagenous colitis, whose identical twin is a coeliac patient. She was investigated because of severe exacerbation of her diarrhoea, and had a poor response to trials of prednisolone and sulphasalazine. Her first jejunal biopsy was normal with a total IEL count of 31%. She had a CIA negative jejunal fluid, the serum IgA anti-gliadin antibody titer was slightly increased, and the sugar permeability test was within a normal range (0.035). After 6 months on a GFD, she had a partial

clinical improvement, with reduction by half in her faecal frequency. She was recommended to stay on her GFD indefinitely.

Mrs HJ. This 22-year-old very thin woman had a long history of fatigue, constipation, lower abdominal pain and tenesmus. Coeliac disease was suspected because of a duodenal biopsy performed in another hospital, showing a mild PVA, inflammatory cell infiltrate and increased IELs. A rectal biopsy also showed a solitary ulcer. A jejunal biopsy performed at this hospital was morphologically normal, an IEL count of 38%, no $\gamma\delta$ + cells in epithelium, and no significant inflammation. She had a CIA negative jejunal fluid, serum anti-gliadin antibodies were not detected, and the sugar test was slightly abnormal (0.040). The clinical response to GFD was equivocal, though she felt better. A second jejunal biopsy was normal with an IEL count of 28%, a repeated sigmoidoscopy showed no evidence of ulceration, and the rectal biopsy was normal. Her sugar permeability test (0.020) and other bowel functions were normal. She has been recommended to maintain her GFD indefinitely.

Mrs JS. This 22-year-old woman presented with a 4-month history of recurrent mouth ulcers and angular stomatitis, intermittent diarrhoea and abdominal bloating. She has had bowel complaints all her life, with a tendency to

constipation, and she had recently lost some weight and felt tired. Her first jejunal biopsy was morphologically normal with a high IEL count (73%), and had no epithelial $\gamma\delta$ + cells. The sugar permeability test was normal (0.021). She had a CIA negative jejunal fluid, and serum anti-gliadin antibodies were low. She responded clinically to a GFD, though the jejunal biopsy was still slightly abnormal with an IEL count of 56%, and her sugar test was normal (0.014). She has defaulted twice to follow up appointments.

Mrs AS. This 62-year-old woman had a several year history of watery diarrhoea. She had old rickets, faecal incontinence, and had had a previous cholecystectomy and hysterectomy. The first duodenal endoscopic biopsy was normal with an IEL count of 29%, and a jejunal biopsy showed a normal morphology with an IEL count of 35%, but a lamina propria cell infiltrate. Her jejunal fluid was CIA negative, serum anti-gliadin antibodies were not detected, but the sugar permeability test was abnormal (0.046). She had a good response to a GFD, with remission of diarrhoea within one week. Three months later, a second jejunal biopsy showed a normal histology and an IEL count of 15%. This time, frozen sections were available for $\gamma\delta$ + cells in epithelium, which were absent. The sugar permeability test was slightly abnormal (0.042). She remains on a GFD, with good appetite and weight

increased.

Mrs JF. This 49-year-old woman presented with diarrhoea for 3 weeks, lower abdominal pain and weight loss. Her sister has coeliac disease. She had a diagnosis of coeliac disease based on jejunal biopsy with mild chronic inflammation, an IEL count of 42% but no $\gamma\delta$ + IELs. Her sugar permeability test was abnormal (0.052). She responded clinically to a GFD. One year later, she was re-admitted because of the recurrence of the original symptoms. Further investigations revealed a normal rectal biopsy, an active chronic gastritis in stomach, minimal changes in jejunum (IEL count of 28%, no $\gamma\delta$ + IELs), and a colonic chronic inflammatory infiltrate compatible with microscopic colitis. Her sugar test was borderline (0.042), and she had a CIA negative jejunal fluid on both occasions. Within 2 weeks of re-introducing gluten in her diet, she had an episode of watery diarrhoea, which confirmed her clinical sensitivity. She was recommended to continued a strict GFD for the rest of her life. At the time of completion of this thesis this patient is on the list for biopsy.

Mr AB. This 27-year-old man had a 10 year history of lower abdominal pain and watery diarrhoea, which alternated with episodes of constipation. His jejunal biopsy showed minimal changes, with a high IEL count

(75%) and an increased number of $\tau\delta$ IELs (7.1/mm). The sugar permeability test was grossly abnormal (0.173). The ileal function (SeHCAT test) was also abnormal, suggesting a moderately severe bile acid malabsorption. He has been found to be α -1 anti-trypsin deficient (phenotype PiSZ). He had a CIA negative jejunal fluid and serum antigliadin antibodies were not detected. The provisional diagnosis was idiopathic diarrhoea. When he was reviewed one year later, the symptoms persisted, and a trial of GFD was recommended. At the time of completion of this thesis, this patient is on the list for a second biopsy.

TABLE X.1. DETAILS OF PATIENTS TREATED WITH GLUTEN-FREE DIET.

SYMPTOMS AND CLINICAL DIAGNOSIS	JEJUNAL BIOPSY ON NORMAL DIET HISTOLOGY IEL COUNT (/100 enteroc.)	SUGAR PERMEABILITY TEST	CLINICAL EFFECT OF GLUTEN-FREE DIET	JEJUNAL BIOPSY ON GLUTEN-FREE DIET HISTOLOGY IEL COUNT (/100 enteroc)	SUGAR PERMEABILITY TEST
CIA Positive			CIA Positive		
CR.Ulcerative colitis, sclerosing cholangitis. Watery diarrhoea after colectomy and formation of pelvic ileal pouch	Minor villus shortening 15	0.240	CR.No effect of 6 months on GFD	Minor villous shortening 16	0.104
DM.Idiopathic diarrhoea	Normal (on 37 g gluten daily) 80	0.012	DM.Diarrhoea resolved within 2 weeks of low gluten diet (5 g/day)	Normal 23	not done
DP.Watery diarrhoea microscopic colitis by colonoscopic biopsy. No response to prednisolone and sulphasalazine	Normal 46	0.032	DP.Diarrhoea resolved within one week	Normal 36	0.032
PK.Idiopathic diarrhoea, fatigue	Normal 61	0.020	PK.Diarrhoea resolved within 2 weeks	Normal 38	not done
KW.Intermittent watery diarrhoea. Iron deficiency anaemia	Normal 44	0.065	KW.Diarrhoea resolved within one week (patient defaulted)	Not done (patient defaulted)	
HS.Idiopathic diarrhoea, iron deficiency anaemia	Normal 21	0.062	HS.Diarrhoea resolved within 3 days	Normal 23	not done
JP.Recurrent mouth ulcers, iron deficiency anaemia. No GI symptoms	Normal 50	0.079	JP.Improvement in mouth ulcers after 2-3 weeks	Not done (proceeded to gluten loading)	0.026
HS.Crohn's disease, clinically inactive. Severe diarrhoea after limited ileal resection	Normal 31	0.035	HS.No effect of two months on GFD	Not done	

TABLE X.1. DETAILS OF PATIENTS TREATED WITH GLUTEN-FREE DIET
(continued)

SYMPTOMS AND CLINICAL DIAGNOSIS	JEJUNAL BIOPSY ON NORMAL DIET HISTOLOGY IEL COUNT (/100 enteroc)	SUGAR PERMEABILITY TEST	CLINICAL EFFECT OF GLUTEN-FREE DIET	JEJUNAL BIOPSY ON GLUTEN-FREE DIET HISTOLOGY IEL COUNT (/100 enteroc)	SUGAR PERMEABILITY TEST
<u>CIA negative</u>					
CS. Identical twin of coeliac. Collagenous colitis. Severe diarrhoea. Poor response to prednisolone and sulphasalazine	Normal 31	0.035	CS. Partial improvement after 6 months on GFD: stool frequency halved	Not done	
HJ. Abdominal pain. Constipation	Normal 38	0.040	HJ. Equivocal response GFD indefinitely	Normal 28	0.020
JS. Recurrent mouth ulcers. Intermittent diarrhoea, tiredness	Min Changes 73	0.021	JS. Improvement of symptoms after 5 months on GFD	Min Changes 54	0.014
AS. Watery diarrhoea Old rickets	Normal 35	0.046	AS. Diarrhoea resolved within one week	Normal 22	0.042
JF. Diarrhoea. Weight loss and abdominal pain	Normal 42	0.052	JF. Initial good clinical response. Recurrence after re- introduction of a normal diet	Min Changes 28	0.042
AB. Watery diarrhoea. Long history constipation and abdominal pain	Min Changes 75	0.173	AB. Waiting for second biopsy Good clinical response (on the list for a second biopsy: 13.12.93)		

SECTION FOUR

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER XI. GENERAL DISCUSSION AND CONCLUSIONS

Inappropriate immunity to gliadin without mucosal histological changes may be more common than previously realized. Between 15 and 20% of the non-coeliac patients we studied were CIA positive, and some of them had a high IEL count. Almost 40% of patients had one or more immunological indices of potential coeliac disease. CIA positive patients are certainly gluten sensitized in one sense, because they produced high titers of specific anti-gliadin antibodies. A high IEL count suggests the existence of an enteropathy, which is restored to normal after gluten restriction. They are, by definition, gluten-sensitive. High $\gamma\delta$ IEL counts have a positive correlation with the CIA pattern, but these cells are related to the genetic markers of the disease (Holm 1993), rather than the dietary status.

Studies of jejunal fluid antibodies, and counts of total and $\gamma\delta$ IELs, may be a useful screening test to determine patients suitable for follow-up to determine latent coeliac disease. These may also include cases with biopsies unsuitable for histological assessment, patients with late relapse after gluten challenge, or those in

whom a proper biopsy was not taken before treatment. An abnormal immune activity in mucosa may affect the intestinal function even before morphological changes are observed. Most patients for diagnostic jejunal biopsy are referred because of small bowel symptoms, but some also have nutritional deficiencies, sometimes increased by poor intake, blood loss or metabolic stress (eg infection, pregnancy). Previous work on latent coeliac disease has not considered the possibility of latent coeliac disease in asymptomatic patients.

Diagnostic criteria of latent coeliac disease are stringent, and the diagnosis is usually made in retrospect or by chance. Thus we have suggested that the term **potential coeliac disease** may be more appropriate in clinical practice, to be used while dietary manipulations are undertaken in patients suspected to be latent coeliacs (Ferguson 1993). If the findings are positive, the diagnosis of low-grade coeliac disease is confirmed.

Coeliac and DH patients whose intestinal lesions have resolved on a GFD and whose jejunal biopsies are classified as "normal" for diagnostic purposes may still express subtle pathological or immunological abnormalities similar to those of untreated coeliacs. These include a high count of villous IEL (Ferguson & Murray 1971), an increased expression of the $\gamma\delta$ T cell

receptor by IELs (Savilahti 1990), abnormal intestinal permeability (Bjarnason 1983), and high levels of IgM anti-gliadin antibody, other class antibodies, and IgA anti-gliadin antibody in specimens of jejunal fluid and whole gut lavage fluid (O'Mahony 1991a).

All of these features can be used as putative markers of latent coeliac disease. TCR $\gamma\delta$ + IELs have been found increased in DH patients and in a case of latent coeliac disease detected during a family study (Savilahti 1990, 1992; Maki 1991a; Holm 1993a-b). High ARA and AEM titres are present in some healthy coeliac relatives (Maki 1990). Intestinal permeability changes have also been reported in first-degree relatives of coeliacs (Bjarnason 1983). Up to 30% of coeliac relatives with normal biopsy histology had high $\gamma\delta$ + IEL counts, and this is associated with the HLA-DQ α/β coeliac associated gene markers.

A new method of identifying potential cases is presented in this thesis, based on the observation that the intestinal antibody pattern of coeliac disease - CIA - also occurs in DH patients with normal biopsy histology on a gluten-containing diet (O'Mahony 1990a), and it may provide a diagnostic index of latent coeliac disease in other situations. The CIA pattern may only identify potential coeliac cases, because gluten sensitivity still requires clinical monitoring on a GFD and after a gluten

challenge. It is important to emphasize that a high density of villus IELs may be the only pathological expression of gluten sensitivity in coeliac patients (Marsh 1989a), defining a mild but significant enteropathy, rather than a truly latent condition. Thus a positive result for total IEL count may have different implications compared with positive findings for the other parameters examined.

The approach to the recognition of latent coeliac disease in this thesis differs from other similar studies in the group of patients concerned. Other reports have targeted specific groups, particularly first-degree relatives of coeliacs, which have an expected high incidence of positivity for the coeliac HLA-associated haplotypes. However, the scope of this study has been much wider, since the only selective criteria used is referral for diagnostic small bowel investigations.

Coeliac disease is a life-long condition, and latent coeliac disease refers to those patients with a genetic predisposition, in which the disease is not manifested by enteropathy at the time of the study. The best example is DH patients with normal jejunal biopsies, and they provide the only well defined group of patients in whom in-vivo dietary manipulations (the current gold standard) lead to the development of a gluten-sensitive enteropathy

in many cases. The existence of latent coeliac disease implies that the finding of a normal jejunal biopsy does not completely exclude gluten sensitivity, and patients may be identified by the expression of immunological abnormalities, similar to those which persist in treated coeliac and DH patients after healing of the enteropathy.

There was a positive correlation between high counts of IELs and the expression of HLA-DR antigens by crypt enterocytes in patients with morphologically normal mucosa. This suggests that amongst the range of immunological abnormalities described in these patients, a high total IEL count may identify cases more likely to have a mild DTH reaction in the intestine, whereas other abnormalities (ie. CIA pattern, high $\gamma\delta^+$ IEL counts) are not related with an active process in the intestine.

Two interesting angles emerge from this study, and both affect the management of patients referred for diagnostic jejunal biopsy. First, there is the need to investigate those patients found to be positive for $\gamma\delta^+$ expression by IELs, CIA pattern, etc; probably in connection with the study of other genetic markers associated with the HLA-DQ locus. Second, the selection of patients who need to undergo a trial of GFD will require a complete re-think, with the consequent revision of the diagnostic criteria of the disease. Patients positive for at least two

markers and the expression of the HLA-DQ α / β heterodimer, should be followed up. The diagnosis of latent coeliac disease would be confirmed earlier by a clinical trial of gluten sensitivity in order to introduce a GFD in sensitized patients.

Further elucidation of the relationships between these immunological phenomena and the clinical entity of coeliac disease will require not only direct, in vivo investigations of gluten sensitivity in potential coeliac patients, but also characterization of the genetic make-up of both coeliac and non-coeliac patients, who do or do not have the immune abnormalities described above.

CONCLUSIONS

1. Untreated coeliacs have high levels of IgA and IgM antibodies in intestinal secretions; the pattern is characterized by a polyclonal increase of IgM antibodies.
2. This antibody pattern is maintained in coeliac patients after treatment and healing of the intestinal mucosa. DH patients without enteropathy also have a similar pattern in jejunal fluid.
3. Some non-coeliac symptomatic patients referred for

diagnostic jejunal biopsy have a "coeliac-like intestinal antibody" - CIA - pattern in jejunal fluid, and they may be latent coeliacs.

4. These non-coeliac patients may express other putative markers of latent coeliac disease, such as high total IEL counts and a high proportion of TCR $\gamma\delta$ + IELs. Patients positive for any of these markers should be described as "potential coeliacs".

5. A positive CIA pattern is correlated with high numbers of $\gamma\delta$ T cells in epithelium in non-coeliac patients.

6. Patients positive for 3 markers: the CIA pattern, high counts of total IELs and $\gamma\delta$ + IELs, may be latent coeliacs; as has been shown in the case who later developed an enteropathy after a trial of gluten loading.

7. Some of these symptomatic non-coeliac patients responded clinically to the trial of a gluten-free diet.

8. Non-coeliac patients with morphologically normal biopsy but a high IEL count more frequently expressed HLA-DR antigens in jejunal crypt epithelium than those patients with normal counts, suggesting the existence of an active "low-grade" DTH reaction in mucosa.

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APPENDIX.

PUBLISHED PAPERS

Dissociation between systemic and mucosal humoral immune responses in coeliac disease

S O'Mahony, E Arranz, J R Barton, A Ferguson

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Dissociation between systemic and mucosal humoral immune responses in coeliac disease

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Abstract

We examined humoral immunity in coeliac disease as expressed in serum (systemic immunity), and in saliva, jejunal aspirate, and whole gut lavage fluid (mucosal immunity). The aims were to define features of the secretory immune response (IgA and IgM concentrations and antibody values to gliadin and other food proteins measured by enzyme linked immunosorbent assay (ELISA)) in active disease and remission, and to establish whether secretions obtained by relatively non-invasive techniques (saliva and gut lavage fluid) can be used for indirect measurements of events in the jejunum. Serum, saliva, and jejunal aspirate from 26 adults with untreated coeliac disease, 22 treated patients, and 28 immunologically normal control subjects were studied, together with intestinal secretions obtained by gut lavage from 15 untreated and 19 treated patients with coeliac disease and 25 control subjects. Jejunal aspirate IgA and IgM and gut lavage fluid IgM concentrations were significantly raised in patients with untreated coeliac disease; the lavage fluid IgM concentration remained higher in patients with treated coeliac disease than in controls. Serum and salivary immunoglobulin concentrations were similar in the three groups. Patients with untreated coeliac disease had higher values of antibodies to gliadin compared with treated patients and control subjects in all body fluids tested; these were predominantly of IgA and IgG classes in serum, and of IgA and IgM classes in jejunal aspirate and gut lavage fluid. Values of salivary IgA antibodies to gliadin were significantly higher in untreated coeliacs, though antibody values were generally low, with a large overlap between coeliac disease patients and control subjects. In treated patients, with proved histological recovery on gluten free diet, serum IgA antigliadin antibody values fell to control values, though serum IgG antigliadin antibody values remained moderately raised. In contrast, there was persistence of secretory antigliadin antibodies in treated patients (particularly IgM antibody) in both jejunal aspirate and gut lavage fluid. Antibody responses to betalactoglobulin and ovalbumin were similar to those for gliadin, including persistence of high intestinal antibody values in patients with treated coeliac disease. There was a positive correlation between antibody values in jejunal aspirate and gut lavage fluid, but not between saliva and jejunal aspirate; thus salivary antibodies do not reflect intestinal humoral immunity.

Systemic humoral immunity in coeliac disease has been the subject of intensive investigation.

Numerous studies have established that patients with untreated coeliac disease have high values of circulating antibodies to wheat derived proteins such as gliadin, and that antibody values fall after a period of treatment with a gluten free diet.^{1,4} Estimation of serum IgA antigliadin antibody is now routinely used both as a screening test for coeliac disease and as a means of assessing dietary compliance.

In contrast, information on mucosal immunity in coeliac disease is patchy. There have been many studies of mucosal lymphoid cells^{5,6} and there is circumstantial evidence of a local cell mediated immune response to gluten.^{7,8} Several studies have carefully mapped the numbers of Ig producing plasma cells in the jejunal mucosa of patients with untreated and treated coeliac disease,^{9,10} showing that untreated patients have increased numbers of IgA and IgM (and to a lesser extent, IgG) jejunal plasma cells. In the 1970s, the presence of intestinal antibodies to food antigens was recognised by a relatively insensitive precipitin technique,¹¹ but there are only two studies published,^{12,13} both in children, on the isotype of antibodies to dietary antigens in intestinal secretions, and these give conflicting results.

The general objectives of this study were twofold. In relation to coeliac disease, our aim was to characterise, *in vivo*, intestinal humoral immunity. Total immunoglobulins and specific antibodies to gliadin and to two antigens which are not toxic in coeliacs were measured in three different mucosal secretions. Untreated and treated patients with coeliac disease were studied to determine whether abnormalities of secretory immunity are permanent and intrinsic to the coeliac diathesis or are only present in active disease. Separately, and of relevance to the clinical investigation of mucosal immunity, we studied the relations between systemic and intestinal antibodies, and we examined our data to establish whether patterns of immunoglobulins and antibodies in jejunal fluid are mirrored in other secretions which can be obtained without intubation. The salivary glands are considered part of the common mucosal immune system,¹⁴ and we therefore studied pure parotid saliva. We also used a whole gut lavage technique for the non-invasive collection of intestinal secretions.¹⁵

Methods

PATIENTS STUDIED AT THE TIME OF JEJUNAL BIOPSY

Specimens of saliva, jejunal aspirate, and serum were collected at the same time as jejunal biopsy on 76 occasions in 69 patients. There were 41 patients with coeliac disease (seven studied twice), (23 women and 18 men; median age 42 years, range 15-78) and 28 control patients (14

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women, 14 men, median age 35 years, range 14–75). Control subjects had jejunal biopsy to exclude coeliac disease – jejunal histology in these patients was normal, no other significant pathology was found, and a final diagnosis of functional bowel disease was made. Twenty six of the patients with coeliac disease were untreated and histological examination of the jejunal biopsy specimens showed subtotal or severe partial villous atrophy. Seven of these and a further 15 patients with treated coeliac disease (all with previous diagnostic biopsy specimens) underwent biopsy again while on a gluten free diet. The median period on gluten free diet was three years (range 3 months – 17 years). Eleven had entirely normal jejunal histology (all of these had been taking a gluten free diet for at least two years), and 11 had minor histological changes – for example increased intraepithelial lymphocytes (most patients in this group had been taking a gluten free diet for less than one year).

PATIENTS STUDIED BY WHOLE GUT LAVAGE

Gut lavage was carried out in 15 untreated coeliac disease patients, 19 with treated disease, and 25 control patients. These included 10, eight, and two patients respectively from each group who had also had collection of jejunal aspirate. The median period on a gluten free diet in patients with treated coeliac disease undergoing gut lavage was eight years (range 3 months – 19 years). Eleven of the patients on a gluten free diet had in the past shown a clinical and histological response to the diet, but did not undergo biopsy again at the time of this study. Gut lavage was carried out in 25 control patients (16 women and nine men, median age 52, range 21 – 92 years). These subjects were either healthy volunteers or patients with functional bowel disorder.

SPECIMEN COLLECTION AND PROCESSING

Saliva: parotid salivary flow was stimulated with 5% citric acid sublingually in four 0.5 ml aliquots over five minutes, and collected via a Carlsson-Crittenden cup placed over the parotid duct, with gentle aspiration to maintain position and suction. We collected stimulated saliva only.

Jejunal aspirate: samples were collected from a point just distal to the duodenal-jejunal junction, through the tubing of the Crosby capsule, before taking the biopsy specimen. The protease inhibitor phenylmethyl sulphonylfluoride (PMSF, Sigma) 100 mM in 95% alcohol (20 µl per ml of aspirate) was added before aliquoting.¹⁶ Serum was obtained from all patients.

Gut lavage: the lavage fluid used was a polyethylene glycol (PEG) based electrolyte lavage solution (Golytely). After an overnight fast, patients drank this solution at a rate of 250 ml every 15 minutes for a period of four hours, making the total volume consumed four litres. Specimen collection began once the material passed per rectum became liquid, clear, and free of faecal material. Approximately 200 ml was collected and filtered into 50 ml polypropylene tubes; specimens were centrifuged and treated with protease inhibitors as described by Gaspari *et al.*¹⁵

All the above specimens were aliquoted and stored at –70°C.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Reference materials and reporting of results

For assays of immunoglobulins in the various secretions, serial twofold dilutions of a standard preparation were used to produce a standard curve. For example, for IgA assays dilutions ranging from 1250–19.35 ng/ml of a human colostral IgA standard (Sigma) were used in each test run. Serial dilutions of test samples (varying in initial dilution depending on the type of specimen) were also assayed. Only when the optical density results of at least two of these sample dilutions fell within the range of the standard curve was the assay considered technically satisfactory. The IgA content of the sample was then determined by taking the mean IgA content of these two sample dilutions. For total IgM and IgG in secretions human reference serum (Protein Reference Unit, Sheffield) was used as a standard.

In the assays of specific antibodies, experiments were carried out to define optimal test conditions for each antigen, isotype, and secretion. Serum from a patient with untreated coeliac disease, previously recognised as having high titres of antibodies of all isotypes to a wide variety of dietary antigens, was used as a reference standard. The reference specimen and test specimens were studied at suitable dilutions, varying for the different assays, and the plates were read when the optical density for the standard reached 1.0. Results for test specimens are expressed as optical density readings, % of this standard. Results are thus expressed as non-parametric data; antibody values are not directly proportional to the antigen binding capacity of the sample. This is a feature of all such assays.

Immunoglobulins (jejunal aspirate, gut lavage fluid, and saliva)

Assays were performed in 96 well microtitre ELISA plates (Dynatech). All reactants were added in volumes of 0.125 ml per well and all washes were done three times using saline with 0.05% Tween-80 added. For the assay of total IgA, wells were coated with 100 ng/ml affinity purified goat antihuman IgA (Northeast Labs) in 0.1 M carbonate buffer, pH 9.6, and incubated overnight at 4°C and washed. After washing, serial twofold dilutions of standard and samples (initial sample dilution 1/100) were added to the coated wells. Plates were incubated overnight at 4°C and washed. Goat antihuman IgA conjugated with alkaline phosphatase (Northeast Labs) diluted (in saline with 1% fetal calf serum and 0.05% Tween-80) to a predetermined optimal value was added and plates were incubated for three hours at 20°C. After washing, paranitrophenylphosphate (PNPP, Sigma) 1 mg/ml in 10% diethanolamine (DEA) buffer, pH 9.8, was added. Plates were read at optical density 405 in an MR580 microELISA reader (Dynatech). The IgA content of any given sample was determined as described above.

The method used to determine total IgM and IgG in secretions was similar; initial sample dilution was 1/25.

Serum immunoglobulins were measured by autoanalyser using an immunoturbidimetric method.

Food antibodies

The assay was similar to that described above. Immulon 2 (129B) ELISA plates (Dynatech) were used. Wells were coated with antigen (gliadin, betalactoglobulin and ovalbumin) at a concentration of 5 µg/ml. Betalactoglobulin and ovalbumin were supplied by Sigma; gliadin was supplied by Dr Stefan Strobel. Reference standard and samples were added in duplicate dilutions to the coated wells. The following sample dilutions were used: serum: 1/100 (IgA and IgM) and 1/200 (IgG); jejunal aspirate: 1/10; gut lavage fluid: 1/2; saliva: 1/2. An appropriate dilution of standard was included in each assay; these dilutions gave optimal optical density readings.

We established that these antibodies were specific by incubating samples with the relevant

antigen and showing that the antibody was specifically absorbed out. In a study of 20 samples, the within plate optical density coefficient of variation was 7.3%, and the between plate optical density variation was 11.1%. If the optical densities of the duplicate sample dilutions varied by >15%, the assay was repeated.

STATISTICAL METHODS

Differences in antibody values and immunoglobulin content were analysed using the Mann-Whitney U test. For correlations, Spearman's test was used.

Results

IMMUNOGLOBULIN CONCENTRATIONS (TABLE I)

Serum

No significant differences were observed.

Jejunal aspirate

Untreated coeliac disease patients had significantly higher jejunal aspirate concentrations of IgA, IgM, and IgG compared with controls, and higher IgM compared with patients with treated coeliac disease. Values for treated coeliac disease patients were not significantly different from those of control subjects.

Gut lavage fluid

IgM content was significantly higher in both untreated and treated coeliac disease patients than in control subjects.

Saliva

No significant differences were observed.

TABLE I Immunoglobulin concentrations (median (range)) in serum (mg/ml), jejunal aspirate, gut lavage fluid, and saliva (µg/ml)

	Ig class	Untreated coeliac patients	Treated coeliac patients	Control subjects
Serum	IgA	2.4 (1.3-3.8)	2.0 (0.7-3.3)	1.8 (0.9-4.1)
	IgM	1.1 (0.3-3.4)	0.9 (0.3-3.1)	1.3 (0.3-4.5)
	IgG	10.5 (5.6-15.7)	10.7 (5.4-20.1)	9.5 (7.3-15.9)
Jejunal aspirate	IgA	290* (25-2282)	140.7 (20-1558)	102.5 (22.9-540.6)
	IgM	29.8*† (1.9-357.9)	9.6 (0-174.4)	4.5 (0-39.5)
	IgG	23.9* (3-261.8)	11.8 (0.3-271.4)	7.3 (1.2-29.3)
Gut lavage fluid	IgA	146.2 (10.7-487.5)	90.8 (8.8-621.8)	139.7 (7.9-403)
	IgM	17.5* (2.1-100.1)	25.7* (0-192.2)	5.3 (0-37)
	IgG	2.7 (0.1-34.9)	0.9 (0.1-11.4)	1.0 (0-12.2)
Saliva	IgA	123.8 (48.6-454)	118.1 (32.2-838.2)	165.8 (66.5-606.9)
	IgM	1.2 (0.1-138.9)	1.4 (0.1-12.4)	1.1 (0.1-3.6)
	IgG	1.3 (0-25.1)	0.9 (0.1-10.6)	0.5 (0-34.3)

Immunoglobulin concentration significantly higher ($p < 0.05$) than in control subjects.

Immunoglobulin concentration significantly higher ($p < 0.05$) than in treated coeliac disease patients.

TABLE II Serum food antibody values (median (range))

Antigen	Ig class	Untreated coeliac patients (n=26)	Treated coeliac patients (n=22)	Control subjects (n=28)
Gliadin	IgA	43.7*† (4.6-150)	5.8 (0.3-41.9)	5.3 (0-44.5)
	IgM	43.0 (19.3-95.4)	38.6 (10.5-87.6)	47.9 (16.1-108.8)
	IgG	82.4*† (9.8-137.4)	41.6* (6.8-107.4)	21 (0-105.2)
BLG	IgA	14.7*† (1.4-150)	6.6* (2.1-87.5)	3.3 (0.2-36.4)
	IgM	33.6 (9-125.5)	38.7 (11.3-94.6)	27.4 (3.5-82.2)
	IgG	94.7* (5-150)	70.2 (8.6-150)	30.9 (0-131.8)
OVA	IgA	18.8* (2.6-150)	14.8 (4.8-60.6)	11.4 (0.9-35.9)
	IgM	31.1 (4-87)	37.8 (9.5-123.4)	30.0 (5.7-102.6)
	IgG	64.4 (2.6-150)	63.4* (8.4-150)	27.6 (3.6-150)

Antibody values significantly higher ($p < 0.05$) than in control subjects.

Antibody values significantly higher ($p < 0.05$) than in treated coeliac disease patients.

BLG = betalactoglobulin; OVA = ovalbumin.

TABLE III Jejunal aspirate antibody

Antigen	Ig class	Untreated coeliac patients (n=26)	Treated coeliac patients (n=22)	Control subjects (n=28)
Gliadin	IgA	26.5*† (0.7-134.5)	4.0* (0.3-34.2)	1.1 (0-13.3)
	IgM	80.5*† (0.1-150)	19.6* (2.1-114.4)	1.9 (0-11.7)
	IgG	1.8*† (0-23.4)	1.0* (0-4.8)	0.2 (0-2.2)
BLG	IgA	25.3* (0.6-150)	9.2 (1.1-110.9)	5.0 (0.1-60.8)
	IgM	16.4* (0-150)	9.2* (1.1-78.3)	1.6 (0-11.7)
	IgG	4.5* (0-104)	1.6 (0-35.1)	1.0 (0-7.7)
OVA	IgA	15.8* (4-150)	19.6* (1.5-66.5)	4.2 (0-100.5)
	IgM	18.7* (0-97)	8.4* (0.1-52.5)	1.1 (0-17.7)
	IgG	1.3* (0-26.2)	1.1 (0-11.7)	0.5 (0-26.5)

Antibody values significantly higher ($p < 0.05$) than in control subjects.

Antibody values significantly higher ($p < 0.05$) than in treated coeliac disease patients.

BLG = betalactoglobulin; OVA = ovalbumin.

Jejunal aspirate (Table III)

There was very little antibody detected in jejunal

Figure 1: Jejunal aspirate IgA and IgM antigliadin antibody values. (Median antibody values shown as horizontal bars.)

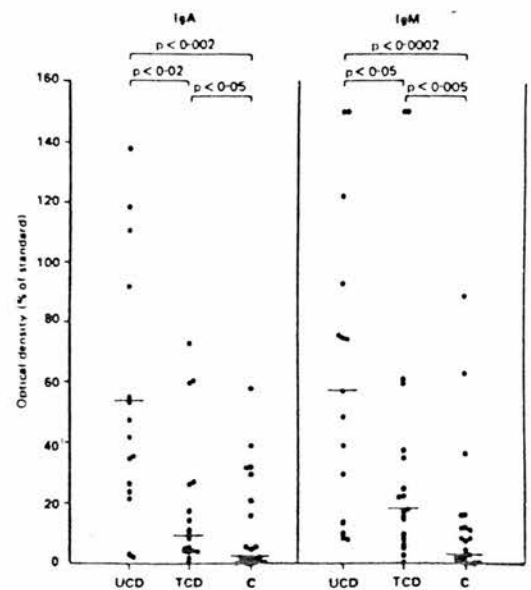
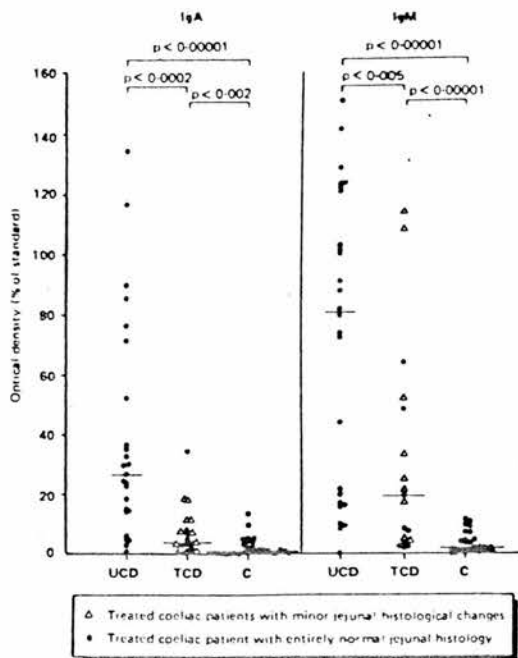


Figure 2: Gut lavage fluid IgA and IgM antigliadin antibody values. (Median antibody levels shown as horizontal bars.)

aspirates from control subjects, but as detailed in Table III, for all three isotypes and all three antigens studied, antibody values were significantly higher in jejunal aspirates from untreated patients than from control subjects (p values all < 0.02). For antigliadin antibodies, values in treated coeliac patients were intermediate between untreated coeliac disease patients and control subjects and significantly different from both. When antibody values in the 11 treated patients with entirely normal jejunal histology were compared with those in control subjects, IgA antigliadin antibody values were not significantly higher. Conversely, IgM antigliadin antibody values remained significantly raised ($p < 0.005$) in this group. Antibodies to betalactoglobulin and ovalbumin showed a greater over-

lap between values in coeliac disease patients and control subjects, but again high IgM antibody values persisted in the treated patients.

Jejunal aspirate IgA and IgM antigliadin antibody values are shown in Figure 1.

Gut lavage fluid (Table IV)

As in jejunal aspirate, high values of IgA and IgM antibodies to gliadin were found in both untreated and treated coeliac disease patients compared with control subjects, with significantly higher antibody values in the untreated compared with the treated patients. High values of IgA and IgM antibodies to betalactoglobulin and ovalbumin were found in untreated patients; high values of IgA and IgM anti-ovalbumin and IgM anti-betalactoglobulin antibodies persisted in the treated coeliac disease patients.

Gut lavage fluid IgA and IgM antigliadin antibody values are shown in Figure 2.

TABLE IV Gut lavage fluid antibody values

Antigen	Ig class	Untreated coeliac patients (n=15)	Treated coeliac patients (n=19)	Control subjects (n=25)
Gliadin	IgA	53.6* (2.2-137.8)	9.2* (0.4-72.9)	2.2 (0-57.9)
	IgM	56.9* (8-150)	17.9* (0.2-150)	3.1 (0-88.7)
	IgG	0.4* (0.2-19)	0.6 (0-3)	0.2 (0-6.5)
Betalactoglobulin	IgA	7.9* (1.8-85.5)	8.4 (0.6-76.5)	3.5 (0.1-71.2)
	IgM	5.6* (0.14-5)	5.3* (0-53)	0.1 (0-22.6)
	IgG	0.7 (0-21)	0.4 (0-8.1)	0.4 (0-4.4)
Ovalbumin	IgA	15.0* (3.7-39.3)	14.7* (0.5-46.8)	4.8 (0-76)
	IgM	6.6* (0.8-33.3)	3.6* (0.46-3)	0.9 (0-20.6)
	IgG	0.3* (0-1.3)	0.0 (0-61.2)	0.0 (0-3.6)

*Antibody values significantly higher ($p < 0.05$) than in control subjects.

†Antibody values significantly higher ($p < 0.05$) than in treated coeliac disease patients.

TABLE V Salivary antibody values

Antigen	Ig class	Untreated coeliac patients (n=26)	Treated coeliac patients (n=22)	Control subjects (n=28)
Gliadin	IgA	5.1* (0.1-29.6)	4.2 (0-12.5)	3.0 (0-11.7)
	IgM	5.1 (0-150)	5.4 (0-35.9)	2.4 (0-15.5)
	IgG	0.3* (0-17.9)	0.0 (0-4.6)	0.0 (0-7.6)
Betalactoglobulin	IgA	8.3* (1.9-53.7)	8.3* (0.46-5)	5.3 (0-29.8)
	IgM	1.0 (0-28.3)	1.2 (0-14.4)	0.8 (0-11.9)
	IgG	0.4 (0-5.8)	0.0 (0-18.8)	0.0 (0-2.9)
Ovalbumin	IgA	15.8 (1.9-92.9)	13.2 (0.6-34)	14.3 (3.4-30.2)
	IgM	1.2 (0-34.1)	1.4 (0-16.7)	1.3 (0-13.3)
	IgG	0.0 (0-13)	0.0 (0-3.1)	0.0 (0-1.7)

*Antibody values significantly higher ($p < 0.05$) than in control subjects.

†Antibody values significantly higher ($p < 0.05$) than in treated coeliac disease patients.

G = betalactoglobulin; OVA = ovalbumin.

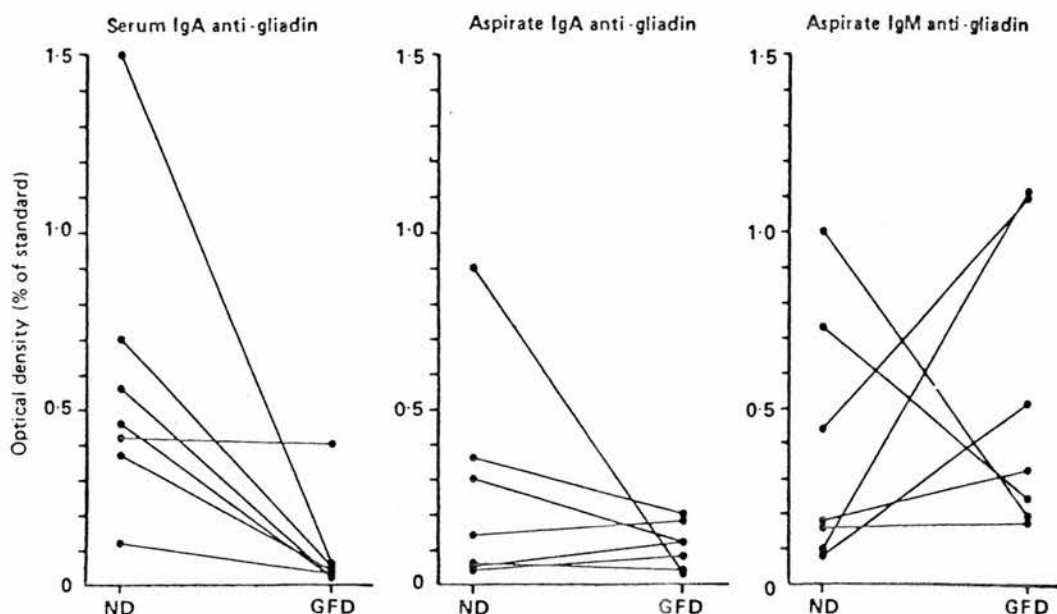
Saliva (Table V)

Salivary antibody values were generally low, with large overlaps between patient groups. Untreated coeliac disease patients had higher values of IgA and IgG antigliadin antibodies compared with control subjects; only IgA antigliadin antibody values were higher than in the treated patients. Higher values of IgA anti-betalactoglobulin antibody were found in both untreated and treated coeliac disease patients compared with control subjects.

SERIAL STUDIES IN COELIAC DISEASE PATIENTS

Seven coeliac disease patients who had a clinical and histological response to a gluten free diet were studied before and after treatment. Serial changes in serum IgA antigliadin antibody and jejunal aspirate IgA and IgM antigliadin antibodies are shown in Figure 3. Whereas serum antibody values fell significantly ($p < 0.05$) with treatment, there was no significant change in the values of intestinal antibody despite histological

Fig. 3. Serial changes in serum anti-gliadin and jejunal IgA and IgM anti-gliadin antibodies in 7 coeliac disease patients before and after treatment with a normal diet; and after treatment with a gluten free diet.



recovery on a gluten free diet. There was, in fact, a trend towards higher values of IgM antibody after treatment. It should be noted that these seven patients had been taking a gluten free diet for six months or less, which may account for the fact that they behaved differently from the treated coeliac disease patients as a whole, who had significantly lower jejunal aspirate antibody values compared with untreated coeliac disease patients.

CORRELATIONS BETWEEN ANTIBODY VALUES IN JEJUNAL ASPIRATE, GUT LAVAGE FLUID, SERUM, AND SALIVA (TABLE VI)

IgA anti-gliadin antibody values were compared in the various body fluids tested. In the untreated coeliac disease patients, we found a positive correlation between antibody values in serum and jejunal aspirate ($r=0.68$, $p<0.0001$) but in the treated coeliac disease patients this correlation was not maintained as they had high values of jejunal aspirate antibody, whereas serum antibody values were low or absent. In the control group, there was a positive correlation ($r=0.59$, $p<0.001$) between antibody values in serum and saliva. No correlation was found between salivary and jejunal aspirate antibody values in any of the three groups.

Both jejunal aspirate and gut lavage fluid had been collected from 20 patients (from all three patient groups). A positive correlation ($r=0.79$, $p<0.01$) was found between antibody values in jejunal aspirate and gut lavage fluid.

Discussion

Intestinal anti-gliadin antibodies in coeliac disease patients were mainly in the IgA and IgM classes, and significant amounts of IgM antibody persisted in the secretions of treated coeliac disease patients with entirely normal jejunal histology. Secreted IgA antibody was detected only in the subgroup of treated coeliac disease patients with minor histological abnormalities, most of whom had had less than a year's treat-

ment with a gluten free diet. It is possible that the intestinal IgA anti-gliadin antibody and minor histological changes could both be due to continued ingestion of small amounts of gluten. Conversely, intestinal IgM antibody values remained higher than in control subjects, even in patients with completely normal jejunal mucosa who had been taking a gluten free diet for some years. It is likely that minute amounts of gluten (complete compliance to a gluten free diet is difficult to achieve in adults) maintains a local immune response rather than a systemic one. Our finding of a persistent IgM antibody response parallels the finding of a relatively high fraction of IgM plasma cells in treated coeliac disease patients.⁷

We found high values of serum IgA and IgG anti-gliadin antibody in untreated coeliac disease patients. In the treated patients, serum IgA anti-gliadin antibody values were similar to those in controls but serum IgG anti-gliadin antibody values, though significantly lower than in the untreated coeliac disease patients, remained significantly higher than in control subjects. These findings agree with those of other reports.^{17,18}

Concentrations of IgA, IgM, and IgG were all high in jejunal aspirates from untreated coeliac disease patients. Counts of jejunal plasma cells and in vitro immunoglobulin production in coeliac disease are higher than in control subjects for all isotypes,¹⁰ supporting the view that the immunoglobulins (at least IgA and IgM) in coeliac intestinal secretions are produced locally.

TABLE VI Correlation between IgA anti-gliadin antibody values in serum, jejunal aspirate, and saliva

Group	Serum and saliva	Serum and aspirate	Saliva and aspirate
Untreated coeliac disease (n=26)	$r=0.021$ (NS)	$r=0.68$ ($p<0.0001$)	$r=0.293$ (NS)
Treated coeliac disease (n=22)	$r=0.392$ (NS)	$r=0.04$ (NS)	$r=0.323$ (NS)
Control subjects (n=28)	$r=0.593$ ($p<0.001$)	$r=-0.193$ (NS)	$r=0.071$ (NS)

NS=not significant.

It is likely that most of the jejunal IgG is plasma derived as the numbers of IgG secreting plasma cells are low even in untreated coeliac disease.⁹ We should point out that immunoglobulin measured in jejunal aspirate was total immunoglobulin and not specific secretory IgA and IgM. It is possible that at least some of the jejunal IgA and IgM is serum derived (coeliac disease is a protein losing disorder). We are currently characterising jejunal immunoglobulins and antibody in terms of molecular weight and percentage of total Ig which contains a secretory component. An increase in the intestinal fluid immunoglobulin content was not accompanied by equivalent changes in serum immunoglobulins; in fact, the serum IgM content was noticeably low in some untreated coeliac disease patients.

We have not yet assessed the contribution of specific antigliadin antibody to the increase in intestinal immunoglobulin content in coeliac disease. Falchuk and Strober,¹⁹ using an affinity chromatography technique, reported that approximately half of the net increase in IgA and IgM synthesis (in an in vitro model of gluten challenge) was due to synthesis of antigliadin antibody. Conversely, in a more recent report of in vitro secretion of antigliadin antibody by coeliac jejunal mucosal biopsy specimens,²⁰ Ciclitera *et al* calculated that antigliadin antibody accounted for 2.1%, 12.1%, and 4.1% of the total concentrations of IgA, IgM, and IgG respectively.

In any event, enhanced intestinal antibody production in coeliac disease is not limited to gluten derived proteins: we found high values of intestinal antibody to betalactoglobulin and ovalbumin in untreated coeliac disease patients, with persistence of IgM antibody to these proteins in treated patients. Antibodies to these food proteins were less specific to coeliac disease than antigliadin antibodies. It has been suggested that high values of serum antibody to these proteins in untreated coeliac disease is simply the result of increased intestinal permeability to antigens.

Although ELISA is now accepted as the standard assay technique for measuring antibodies to food proteins in coeliac disease, several different ELISA methods have been described, and different reference preparations are used by each group of investigators. The method we used is essentially similar to that of Savilahti *et al*,¹ and we used crude gliadin as antigen for the ELISA (rather than say, alphagliadin), as Skeritt *et al*²¹ have shown that sera and intestinal aspirates from coeliac patients contain antibodies which bind to several different gliadin subunits. We have not ascribed levels of 'positivity' or 'negativity' to antibody values; such arbitrary designations are of some value in screening tests used in clinical practice but not in prospective research investigations.

Although untreated coeliac disease patients had statistically higher values of salivary IgA and IgG antigliadin antibodies compared with controls, salivary antibodies were generally low with a large overlap between patients and controls. Furthermore, there was no correlation between jejunal aspirate and salivary antibody values. Our results do not suggest that antibody tests on

saliva have any diagnostic or screening potential.

On the other hand, gut lavage offers a relatively non-invasive alternative to intubation for the collection of intestinal secretions for immunoglobulin and antibody studies. Antibody findings in gut lavage fluid were broadly similar to those in jejunal aspirate, with a positive correlation for IgA antigliadin antibody values in those patients studied by both techniques. Immunoglobulin concentrations, however, differed considerably in jejunal aspirate and gut lavage fluid; all three immunoglobulin isotypes were raised in jejunal aspirate in the untreated coeliac disease patients, whereas only IgM was significantly raised in gut lavage fluid. Gut lavage fluid is likely to contain secretions from not only the small bowel but also gastric juice, bile, pancreatic secretions, and colonic secretions. In this respect, it is not a homogeneous fluid, unlike jejunal aspirate, which reflects events in the jejunum only, and is thus more likely to represent accurately local immune phenomena in coeliac disease. Fluid flow rate is another factor that may influence Ig values in exocrine secretions; this may partly account for differences in findings in jejunal aspirate and gut lavage fluid.

The relevance of gluten reactive intestinal B cells and antibodies to the pathogenesis of coeliac disease is uncertain; the presence of antibodies to gluten derived proteins in patients with coeliac disease may be merely an epiphenomenon in the context of a T cell mediated enteropathy with expansion of the relevant populations of T helper as well as effector cells.²² It is certainly possible that mucosal IgM antibody is immunopathogenic, for example by fixing complement in the immediate vicinity of enterocytes.

This study shows the dissociation of systemic and intestinal humoral immune responses in patients with coeliac disease, and is evidence that for the study of immunopathology of intestinal disease, direct investigation of the gut is mandatory.

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Latent Coeliac Disease

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Definitions and Diagnostic Criteria of Latent and Potential Coeliac Disease

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Heterogeneity of Coeliac Disease

Within the framework of the current definition (a permanent gluten-sensitive enteropathy), clinical, pathological, epidemiological and immunological approaches are revealing several forms of coeliac disease. In so-called *active coeliac disease*, malabsorption and nutritional deficiencies range from profound to minimal; clinically *silent coeliac disease* is being increasingly recognised, for example in family studies. Pathologically, there is also a degree of heterogeneity. Descriptive terms such as 'flat mucosa', or 'subtotal villus atrophy', are the pathologist's shorthand for a cluster of features (villus, crypt sizes, epithelial cell damage, intraepithelial and lamina propria lymphoid cell infiltrates) which together characterise the enteropathy of coeliac disease. Quantitative histology and computerised image analysis have shown that these features occur in a continuum, with the flat lesion at one end of the spectrum and at the other end a mucosa with normal villus and crypt architecture, but an abnormally high density or count of villus intra-epithelial lymphocytes (IEL) [1, 2]. The latter would be reported as normal by most clinical pathologists.

This fact is important when one reviews the evidence for the existence of *latent coeliac disease*. This term should only be applied to patients who fulfil the following conditions: (i) have a normal jejunal biopsy while taking a normal diet, and (ii) at some other time, before or since, have had a flat jejunal biopsy which recovers on a gluten-free diet.

The suggestion that there might be a 'pre-coeliac' state was first made by Weinstein [3] who described 2 patients with dermatitis herpetiformis (DH) and normal jejunal biopsies in whom typical coeliac-like enteropathy developed some weeks after 20 g of gluten was added to their already gluten-

containing diet. Two studies from the UK confirmed this observation [4, 5] and the concept is supported by case reports of coeliac patients in whom, by chance, a jejunal biopsy has previously been taken and reported as normal [6–8].

If full morphometric analysis were to show changes at the mild end of the spectrum of coeliac-like enteropathy in the original biopsies (as has been reported in 2 such patients [8]), this would require that the descriptive term be revised from *latent* to a more general term such as *low grade* or *mild enteropathy*.

Approaches to the Recognition of Latent or Low-Grade Coeliac Disease

Coeliacs whose intestinal lesions have resolved on GFD and whose jejunal biopsies are classified as 'normal' for diagnostic purposes may still express subtle pathological or immunological abnormalities similar to those of untreated coeliacs. These abnormalities include a high count of villus IEL [9]; increased gamma/delta T cell receptor expression by IEL [10]; abnormal jejunal permeability [11]; and high levels of IgM antigliadin antibody, other IgM class antibodies, and IgA antigliadin antibody, in specimens of jejunal fluid and whole gut lavage fluid, as described below [12].

Thus, one approach to the recognition of potential latent or low-grade coeliacs may be by studies of IEL T cells expressing gamma/delta receptors. This presents logistic problems, as the relevant immunohistochemical studies must be performed on frozen sections. Positive results have been reported in a single case of latent coeliac disease detected during family studies in Finland [7]. We recently reported that there is a characteristic pattern of intestinal fluid antibodies in untreated and treated coeliac disease, which also occurs in DH patients without enteropathy [13]. Studies of intestinal antibodies might facilitate the detection of latent coeliac disease in other situations.

Features of the Coeliac-Like Intestinal Antibody Pattern

It is generally agreed that the intestinal fluid in untreated coeliac disease contains high levels both of IgA and IgM anti-GLI antibody, and that in contrast to serum antibodies, levels of intestinal antibodies remain high after healing of enteropathy in GFD-treated coeliacs [12, 14–16]. In a definitive

study of humoral immunity in coeliac disease, we studied levels of antibodies to three dietary proteins in serum, saliva, jejunal fluid and intestinal secretions obtained by whole gut lavage, from untreated and treated coeliacs, and controls [12].

Concentrations of IgA and IgM were high in jejunal fluid of untreated coeliacs, as was gut lavage fluid IgM concentration. Lavage fluid IgM concentration remained higher than controls in treated coeliacs. Untreated coeliacs had high levels of antigliadin antibodies in all body fluids tested; these were predominantly of IgA and IgG classes in serum, and of IgA and IgM classes in jejunal fluid and gut lavage fluid. In treated coeliacs, with proven histological recovery on a gluten-free diet, serum levels of IgA antigliadin antibody fell to control levels. In contrast, there was a persistence of secretory antigliadin antibodies in treated coeliacs (particularly IgM antibody) in both jejunal fluid and gut lavage fluid. Antibody responses to beta-lactoglobulin and ovalbumin were similar to those for gliadin, including persistence of high intestinal antibody levels in treated coeliacs. Key data are presented in tables 1 and 2.

Thus, we can define a 'coeliac-like intestinal antibody' (CIA) pattern as the presence in jejunal fluid of IgM anti-GLI together with at least two of the three other characteristic antibodies, IgA anti-GLI, IgM anti-OVA and IgM anti-BLG. The significance of a high level of IgM anti-GLI as an isolated finding, remains to be determined.

It is now clear that CIA+ specimens also contain high concentrations of total IgM. Indeed, there is considerable evidence in the literature of over-expression of IgM generally in the intestinal mucosa of coeliacs. Furthermore, in a recent investigation of intestinal immune responses to an enteric vaccine, coeliacs generated high levels of intestinal IgM antibodies to a bacterial antigen, cholera toxin B subunit [unpubl.].

Studies in Dermatitis herpetiformis

We then examined intestinal humoral immunity in 8 patients with DH who had normal jejunal histology (as determined by quantitative morphometry) on a gluten-containing diet, to determine whether such patients, despite the absence of enteropathy, have abnormalities of secretory immunity similar to those encountered in patients with coeliac disease [13]. As a group, the DH cases resembled classical untreated coeliacs in their pattern of secretory immunity with higher than normal concentrations of IgA, IgM and

Table 1. Levels of antibody to gliadin, beta-lactoglobulin and ovalbumin in jejunal fluid, assayed by ELISA (results are expressed as optical density readings, % of standard reference preparation, median and range)

Antigen	Ig class	Untreated coeliac (n = 26)	Treated coeliac (n = 22)	Controls (n = 28)
Gliadin	IgA	26.5 ^{*,*} (0.7-134.5)	4.0 [*] (0.3-34.2)	1.1 (0-13.3)
	IgM	80.5 ^{*,*} (0.1-150)	19.6 [*] (2.1-114.4)	1.9 (0-11.7)
	IgG	1.8 ^{*,*} (0-23.4)	1.0 [*] (0-4.8)	0.2 (0-2.2)
BLG	IgA	25.3 [*] (0.6-150)	9.2 (1.1-110.9)	5.0 (0.1-60.8)
	IgM	16.4 [*] (0-150)	9.2 [*] (1.1-78.3)	1.6 (0-11.7)
	IgG	4.5 [*] (0-104)	1.6 (0-35.1)	1.0 (0-7.7)
OVA	IgA	15.8 [*] (4-150)	19.6 [*] (1.5-66.5)	4.2 (0-100.5)
	IgM	18.7 [*] (0-97)	8.4 [*] (0.1-52.5)	1.1 (0-17.7)
	IgG	1.3 [*] (0-26.2)	1.1 (0-11.7)	0.5 (0-26.5)

* p < 0.05 vs. control; * p < 0.05 vs. treated coeliacs.

IgG, and high levels of specific antibodies (IgA and IgM) to the 3 food proteins. Levels of serum IgA antigliadin antibody in the DH patients were low.

Our finding of a coeliac-like pattern of secretory immunity in DH patients without enteropathy suggested that investigation of gut humoral immunity may provide a diagnostic index of latent coeliac disease.

Two-Stage Model of Coeliac Disease

Considerable effort has been directed towards the development of an animal model of gluten-sensitive enteropathy. Our studies with mice [17] showed that immunological sensitisation to gliadin does not trigger the

Table 2. Levels of antibody to gliadin, beta-lactoglobulin and ovalbumin in whole gut lavage fluid, assayed by ELISA (results are expressed as optical density readings, % of standard reference preparation, median and range)

Antigen	Ig class	Untreated coeliac (n = 15)	Treated coeliac (n = 19)	Controls (n = 25)
Gliadin	IgA	53.6* (2.2-137.8)	9.2* (0.4-72.9)	2.2 (0-57.9)
	IgM	56.9* ⁺ (8-150)	17.9* (0.2-150)	3.1 (0-88.7)
	IgG	0.4* (0.2-19)	0.6 (0-3)	0.2 (0-6.5)
BLG	IgA	7.9* (1.8-85.5)	8.4 (0.6-76.5)	3.5 (0.1-71.2)
	IgM	5.6* (0-14.5)	5.3* (0-53)	0.1 (0-22.6)
	IgG	0.7 (0-21)	0.4 (0-8.1)	0.4 (0-4.4)
OVA	IgA	15.0* (3.7-39.3)	14.7* (0.5-46.8)	4.8 (0-76)
	IgM	6.6* (0.8-33.3)	3.6* (0-46.3)	0.9 (0-20.6)
	IgG	0.3 (0-1.3)	0.0 (0-61.2)	0.0 (0-3.6)

* $p < 0.05$ vs. control; ⁺ $p < 0.05$ vs. treated coeliacs.

development of a T cell-mediated lesion of the intestine when the diet contains gluten. Additional factors, such as those occurring during intestinal anaphylaxis or a graft-versus-host reaction, were necessary. Enhanced antigen presentation, recruitment of specific T cells in the mucosa, up-regulation of the expression of class II antigens and failure of suppression, are all candidate mechanisms for the effects observed.

The proposed two-stage model of coeliac disease [13] derives from the confluence of several lines of clinical and experimental work and can be stated as follows:

Inappropriate immunity to gliadin is a relatively frequent occurrence; it is genetically restricted, and may be expressed not only in gut and skin, but also in the mouth (recurrent aphthae), kidneys (IgA nephropathy) and joints (some arthritides). Expression of T cell-mediated immunity to gliadin in the

gut occurs across a spectrum of histological and functional abnormalities. The minimal lesion may appear histologically normal, or as a virtually normal biopsy with a high count of villus IEL; the fully expressed lesion is a flat mucosa with crypt hyperplasia, typical of coeliac disease.

Mucosal immunological sensitisation is an invariable feature of coeliac disease but is not the precipitating factor for the expression of the full intestinal lesion; a second factor drives the enteropathy from minimal (latent) to overt, either by immunological mechanisms or by direct ancillary effects on enterocytes. Candidate factors include an episode of hyperpermeability, nutrient deficiency, increased dietary gluten, impaired intraluminal digestion of ingested gluten, adjuvant effects of intestinal infection and a non-HLA-associated gene. There may be a complementation of these with a multifactorial trigger mechanism.

Frequency of Patients with the CIA Pattern in a Cohort Referred for Jejunal Biopsy

During the last year we assessed the frequency of the CIA pattern in a prospective study of 140 patients who attended for diagnostic small bowel biopsy [18]. Forty-two were biopsies in coeliac or DH patients, including 14 newly presenting coeliacs; in the 98 non-coeliac cases, studies of jejunal fluid revealed the CIA pattern in 16 cases.

Patients referred to us for jejunal biopsy usually have symptoms suggestive of coeliac disease, and this was the case for the 16 non-coeliac patients with a positive CIA pattern. Diarrhoea was the main complaint in 11, abdominal pain or discomfort in 3 and the other 2 had unexplained and relatively asymptomatic anaemia. The final diagnoses for patients in the group of CIA-positive cases were similar to those in the group who were CIA negative.

Jejunal biopsy histology was reported by a consultant pathologist as normal in 14, mildly abnormal in two. The lactulose/rhamnose permeability test was positive in only 4 cases including the 2 with mild pathological changes; brush-border enzyme assays had been performed in 12 and were entirely normal in 10 cases. Levels of serum IgG antigliadin antibodies (anti-GLI) were normal in 9 cases and of IgA anti-GLI in 10.

However, when formal counts of IEL were performed these were found to be abnormally high in 6 of these 16 patients, 5 with absolutely normal villi and 1 with minor villus blunting.

Objective assessment of gluten sensitivity by treatment with a gluten-free diet will only be possible in those patients who have minor biopsy abnormalities. Three of the 16 CIA+ patients (2 with high IEL count, 1 with mild enteropathy and normal IEL count) have had a trial of gluten restriction; in 2 there was a rapid resolution of watery diarrhoea and in both of these a high IEL count was present in the original biopsy and has fallen to normal after gluten restriction. There was no clinical or pathological effect of 6 months' gluten-free diet in the third patient. A further such patient seen recently has been found to have a high count of gamma/delta IEL, as well as a high total IEL count and a positive CIA pattern.

This work may have considerable therapeutic implications. Sixteen of 98 non-coeliac patients were CIA positive. This is similar to the number of new patients with coeliac disease (14) diagnosed in the course of the study. If further research confirms that many CIA+ patients are gluten-sensitive, then by implication, the previous definition of coeliac disease will have excluded up to half of the symptomatic patients, referred for jejunal biopsy, who would benefit clinically from a gluten-free diet.

Theoretical Aspects

We have not yet established the immunopathogenesis of the CIA pattern. Up-regulation of the IgM component of intestinal immunity could reflect a primary disorder of B cells, aberrant function of immunoregulatory T cells, or the existence of an unusual immunostimulant operating in the gut mucosa. An abnormality of trans-epithelial immunoglobulin transport mechanisms may also operate.

Further work will show the interrelationships between high total IEL count, high gamma/delta IEL counts, and the presence of the CIA pattern in patients with normal biopsy architecture. Either these will turn out to be very sensitive indices of mucosal DTH *expression*, and all associated with production of IL2 and interferon- γ , or their patterns of occurrence may vary in individual patients, perhaps genetically determined, and relevant to the *induction* of a state of aberrant immunity to gluten.

Conclusion

Subtle aberrations from normal occur in the small bowel of most patients with so-called 'latent' coeliac disease, and comprise a high count of villus

Table 3. Proposed nomenclature of forms of coeliac disease and associated conditions

Type of coeliac disease	GI symptoms	Pathology of jejunal biopsy
Active	+	PVA/SVA
Silent	-	PVA/SVA
Latent	usually -	normal or high IEL count
Low-grade or mild enteropathy	+	high IEL count
Potential	+ or -	normal or high IEL count

IEL, increased gamma/delta T cell receptor utilisation by IEL, up-regulation of mucosal IgM responses. One or more of these may be related to the fundamental immunopathogenesis of the disease, i.e. is the factor which leads to induction of abnormal mucosal immunity to gliadin; alternatively, their presence may signal low-grade expression of hypersensitivity. Since clinical gluten sensitivity exists in a proportion of patients with these biopsy features, the present pathological description of coeliac disease should be revised. Treatment with a gluten-free diet (carefully monitored) should be offered to symptomatic patients with these minor forms of enteropathy.

It is likely that all patients with DH have latent or fully expressed gluten-sensitive enteropathy. However, only rarely, and usually by chance (e.g. previous biopsy in a research investigation), do other patients fulfil criteria for latent coeliac disease. For prospective studies of candidate latent coeliacs, such as those with high IEL count, positive CIA pattern, high gamma/delta expression of IEL, relatives of coeliacs, IgA-deficient individuals, a more generally applicable expression is needed. The term *potential* coeliac disease (table 3) is proposed.

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Leading article

Clinical and pathological spectrum of coeliac disease – active, silent, latent, potential

Currently recognised forms of gluten sensitive enteropathy

Within the framework of the current definition (a permanent gluten sensitive enteropathy), clinical, pathological, epidemiological, and immunological approaches are revealing several forms of coeliac disease. In so called active coeliac disease, malabsorption, and nutritional deficiencies range from profound to minimal; clinically silent coeliac disease is being increasingly recognised – for example, in family studies. Pathologically there is also a degree of heterogeneity. Descriptive terms such as 'flat mucosa', or 'subtotal villus atrophy', are the pathologist's shorthand for a cluster of features (villus, crypt sizes, epithelial cell damage, intraepithelial and lamina propria lymphoid cell infiltrates) which together characterise the enteropathy of coeliac disease. Quantitative histology and computerised image analysis have shown that these features occur in a continuum, with the flat lesion at one end of the spectrum and a mucosa with normal villus and crypt architecture, but an abnormally high density or count of villus intraepithelial lymphocytes, at the other.^{1,2} The latter would be reported as normal by most clinical pathologists. This fact is important when the evidence for the existence of latent coeliac disease is reviewed. This term should only be applied to patients who fulfil the following conditions: (i) have a normal jejunal biopsy while taking a normal diet; (ii) at some other time, before or since, have had a flat jejunal biopsy which recovers on a gluten free diet.

The suggestion that there might be a 'precoeliac' state was first made by Weinstein who described two patients with dermatitis herpetiformis and normal jejunal biopsies in whom typical coeliac like enteropathy developed some weeks after 20 g gluten was added to their already gluten containing diet.⁴ Two studies from the United Kingdom have confirmed this observation^{4,5} and the concept is supported by case reports of coeliac patients in whom, by chance, a jejunal biopsy has previously been taken and reported as normal.⁶⁻⁸

Subtle pathological and immunological abnormalities in some latent coeliacs

If full morphometric analysis were to show changes at the mild end of the spectrum of coeliac like enteropathy in the original biopsies (as has been reported in two such patients)⁸ this would require that the descriptive term in these cases be revised from latent to low grade or mild gluten sensitive enteropathy. Furthermore, it would greatly facilitate research and clinical management of such patients if there was a means of identifying them, more widely available and less technically demanding than computerised image analysis.

Coeliacs whose intestinal lesions have resolved on a gluten free diet and whose jejunal biopsies are classified as 'normal' for diagnostic purposes may still express subtle pathological or immunological abnormalities similar to those of untreated coeliacs. These abnormalities include a high count of villus IEL⁹; increased gamma/delta T cell receptor expression by intraepithelial lymphocytes¹⁰; abnormal jejunal permeability¹¹; and high concentrations of IgM antigliadin antibody, other IgM class antibodies, and IgA antigliadin antibody (the

'coeliac like intestinal antibody' pattern) in specimens of jejunal fluid and whole gut lavage fluid.¹²

One approach to the recognition of potential latent or low grade coeliacs is by studies of intraepithelial lymphocyte T cells expressing gamma/delta receptors. This presents logistic problems, as the relevant immunohistochemical studies must be done on frozen sections, but positive results have been reported in a single case of latent coeliac disease detected during family studies in Finland.⁷

We recently reported that the characteristic coeliac like intestinal antibody pattern of intestinal fluid antibodies also occurs in dermatitis herpetiformis patients without enteropathy, a group of patients in whom it is likely that all or most are in fact latent coeliacs.¹³ Similar studies of intestinal antibodies might facilitate the detection of latent coeliac disease in other situations.

Two stage model of coeliac disease

We have proposed a two stage model of gluten sensitive enteropathy, latent and fully expressed.¹³ This derived from the confluence of several lines of clinical and experimental work and can be stated as follows: induction of a state of inappropriate immunity (hypersensitivity) to gliadin is a relatively frequent occurrence, genetically restricted. The effects of abnormal interaction between the immune system and gluten may be expressed not only in gut (coeliac disease) and skin (dermatitis herpetiformis), but also in the mouth (recurrent aphthae), kidneys (IgA nephropathy) and joints (some arthritides). Within the intestinal mucosa, expression of T cell mediated immunity to gliadin in the gut occurs across a spectrum of histological and functional abnormalities. The minimal lesion may appear histologically normal, or as a virtually normal biopsy with a high count of villus intraepithelial lymphocytes; the fully expressed lesion is a flat mucosa with crypt hyperplasia, typical of coeliac disease.

Studies in mice¹⁴ showed that immunological sensitisation to gliadin does not trigger the development of a T cell mediated lesion of the intestine when the diet contains gluten. Additional factors, such as those occurring during intestinal anaphylaxis or a graft-versus-host reaction, were necessary. Enhanced antigen presentation, recruitment of specific T cells in the mucosa, up-regulation of the expression of class II antigens and failure of suppression, are all candidate mechanisms for the effects observed.

By analogy, although mucosal immunological sensitisation is an invariable feature of coeliac disease, it is not the precipitating factor for the expression of the full intestinal lesion; a second factor drives the enteropathy from minimal (latent) to overt, either by immunological mechanisms or by direct ancillary effects on enterocytes. Candidate factors include an episode of hyperpermeability, nutrient deficiency, increased dietary gluten, impaired intraluminal digestion of ingested gluten, adjuvant effects of intestinal infection and a non-HLA associated gene.

Significance of a high count of intraepithelial lymphocytes

In animal work on delayed type hypersensitivity in rodent

testine, a rise in the count of villus intraepithelial lymphocytes was a sensitive and early feature of the expression of mucosal delayed type hypersensitivity¹⁵ and is induced by signals from activated lamina propria CD4 T cells.¹⁶ By analogy, in clinical practice a high count of intraepithelial lymphocytes in an architecturally normal small bowel biopsy will also imply a state of T cell activation, either antigen driven – for example, by gluten, giardia, histocompatibility antigens – or as a result of aberrant mucosal immunoregulation (as in some theories of the pathogenesis of inflammatory bowel disease).

There is now a substantial body of evidence that the expression of gluten hypersensitivity as enteropathy may be minimal, measurable only if a count of intraepithelial lymphocytes is performed. An accepted name for this type of pathology is needed, such as 'high density intraepithelial lymphocyte enteropathy'. This must be clearly differentiated from various other forms of non-coeliac enteropathy, such as that of HIV infection.¹⁷ Further work will show how a high count of intraepithelial lymphocytes relates to gamma delta intraepithelial lymphocyte counts, and whether these are merely sensitive indices of mucosal delayed type hypersensitivity expression, and all associated with production of IL2 and gamma interferon, or whether high density of intraepithelial lymphocytes, the unusual up regulation of IgM and/or gamma delta T cell numbers are independent factors, perhaps genetically determined, relevant to the induction of a state of aberrant immunity to gluten, and thus directly to the pathogenesis of coeliac disease.

Clinical importance of an extension of the pathological criteria for coeliac disease

We have recently assessed the frequency of the coeliac like intestinal antibody pattern, a candidate marker of latent coeliac disease, in patients referred for diagnostic small bowel biopsy. Studies of jejunal fluid revealed the coeliac like intestinal antibody pattern in 16 of 98 non-coeliac cases, of whom six also had a high count of intraepithelial lymphocytes (Arranz and Ferguson, submitted). If further research shows that some of these patients are clinically gluten sensitive (and we already have some evidence to support this), then by implication, the previous definition of coeliac disease (a flat mucosa) may have excluded up to half of symptomatic patients, referred for jejunal biopsy, who would benefit clinically from a gluten free diet. The present pathological description of coeliac disease may need to be revised and treatment with a gluten free diet (carefully monitored)

offered to symptomatic patients with minor forms of enteropathy.

Only rarely, and usually by chance – for example, previous biopsy in a research investigation – does a patient fulfil criteria for latent coeliac disease. A more generally applicable expression is needed to describe people who should have the diagnosis of latent or low grade coeliac disease considered – such as those with high intraepithelial lymphocyte count, positive coeliac like intestinal antibody pattern, high gamma delta expression of intraepithelial lymphocytes, relatives of coeliacs, IgA deficient individuals. The term 'potential coeliac disease' is proposed.¹⁸

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ALIMENTARY TRACT

Intestinal Antibody Pattern of Celiac Disease: Occurrence in Patients With Normal Jejunal Biopsy Histology

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Background: Patients with celiac disease have immunoglobulin (Ig) M antibodies and IgA antigliadin antibody in gut secretions; this pattern of intestinal immunity may be a marker of latent celiac disease. Its frequency in patients referred for jejunal biopsy has been examined. **Methods:** Serum IgG and IgA antigliadin antibody, jejunal fluid IgA and IgM antibodies to gliadin, ovalbumin and β lactoglobulin, and jejunal fluid IgA and IgM concentrations were measured by enzyme-linked immunosorbent assay. **Results:** Seventeen of 19 celiac patients on normal diet and 16 of 23 on gluten-free diet had the celiaclike antibody pattern, as did 41 of 217 other patients. Jejunal biopsy histology had been classified as normal in 38 of these, with minor abnormalities in 3; however, intraepithelial lymphocyte (IEL) counts were high in 13 cases. Trial of a gluten-free diet produced clinical improvement in 6 of 7 antibody-positive patients. After extra dietary gluten, one developed subtotal villous atrophy. **Conclusions:** The celiaclike intestinal antibody pattern and a high IEL count may be markers of latent gluten-sensitive enteropathy; some of these patients are clinically gluten sensitive in the absence of enteropathy.

Celiac disease is currently defined as a permanent intolerance of the small bowel mucosa to gluten.¹ Because there is great variability of malabsorption and heterogeneity of clinical presentation, the diagnosis is based on the presence of a characteristically abnormal jejunal biopsy (total or partial villus atrophy with crypt hyperplasia) while the patient is taking a normal diet, with improvement in histological appearances after treatment with a gluten-free diet (GFD).

This definition is being challenged by the emerging evidence that latent celiac disease² is not particularly rare. Latent celiacs are patients who eat a normal diet and have apparently normal jejunal histology but who at some other time before or since have a gluten-sensitive enteropathy. The concept was first derived from a gluten supplementation experiment in two patients with dermatitis herpetiformis (DH),² and patients

with DH, together with relatives of celiacs, have been the most frequently studied.^{3,4}

Celiacs whose intestinal lesions have resolved on GFD and whose jejunal biopsy samples are classified as "normal" for diagnostic purposes may still express subtle pathological or immunological abnormalities similar to those of untreated celiacs. These include a high count of villus intraepithelial lymphocyte (IEL),⁵ increased γ/δ T-cell receptor expression by IEL,⁶ abnormal jejunal permeability,⁷ and high levels of immunoglobulin (Ig) M antigliadin antibody, other IgM class antibodies, and IgA antigliadin antibody in specimens of jejunal fluid and whole-gut lavage fluid.⁸

We recently reported that this celiaclike abnormality of intestinal antibodies also occurs in DH patients without enteropathy (the celiaclike intestinal antibody [CIA] pattern) and suggested that studies of intestinal antibodies might facilitate the detection of latent celiac disease in other situations.⁹ Therefore, we have assessed the frequency of the CIA pattern in a prospective study of 259 patients who attended for diagnostic small bowel biopsy, and we considered the potential clinical significance of our findings.

Materials and Methods

Patients

This Gastrointestinal Unit provides a regional service for the clinical investigation of gastrointestinal function. During a 16-month period, a total of 151 patients attended for peroral jejunal biopsy, and jejunal fluid was collected at the same time. Samples of jejunal fluid and small bowel mucosa adequate for analysis were obtained from 140 patients (49 male, 91 female) (series A) ranging in age from

Abbreviations used in this paper: BLG, β lactoglobulin; CIA, celiaclike intestinal antibody; DH, dermatitis herpetiformis; ELISA, enzyme-linked immunosorbent assay; GFD, gluten-free diet; GLI, gliadin; IEL, intraepithelial lymphocyte; OTH, patients with other diagnoses (nonceliac/DH); OVA, ovalbumin; PVA, severe partial villus atrophy; TVA, total villus atrophy.

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> 83 years (mean age, 37.2 years). Serum samples were collected from 137 of these patients. Twenty-two of the patients were celiac patients undergoing follow-up biopsies to assess response to GFD; a decision as to the final diagnosis in the remaining 118 cases was made 2–3 months after the biopsy from examination of the case records and without knowledge of the results of antibody tests on serum or secretions. Similar studies were performed in a further cohort of nonceliac patients (series B).

Specimen Collection and Processing

After an overnight fast, the patient swallowed a duodenal biopsy capsule together with 15 mg of Metoclopramide (Beecham; Welwyn Garden City, England). When the capsule had passed to the first loop of jejunum (assessed by fluorograph screening), 1–2 mL intestinal fluid was drained gravimetrically through the tubing. The protease inhibitor phenylmethylsulfonyl fluoride, 20 μ L/mL at a concentration of 100 mg/mL, was immediately added. The capsule was then removed to obtain a jejunal mucosal biopsy. Jejunal fluid was transferred on ice to the laboratory and stored at -70°C in 10 minutes of collection. Venous blood samples were taken and the serum separated, aliquotted, and stored at -70°C .

Jejunal mucosal biopsy specimens were formalin-fixed and sent to the diagnostic pathology laboratory for examination by a consultant pathologist who reported both in a written textual description and made the following classifications: (1) total villus atrophy (TVA), (2) severe partial villus atrophy with crypt hyperplasia (PVA), (3) minor non-specific abnormalities (these included villus shortening without crypt hyperplasia and general increase in inflammatory cell infiltrate), and (4) morphologically normal.

If the specimen was large enough, a separate piece of mucosa was taken for assays of disaccharidases (lactase, sucrase, maltase, and trehalase).¹⁰ In some patients, a lactulose/rhamnose test of jejunal permeability was also performed.¹¹

Immunoglobulin and Antibody Assays

In specimens of jejunal fluid, concentrations of immunoglobulin Ig A and IgM and levels of IgA and IgM antibodies to gliadin (GLI) (gift from Dr. H. Weiser), ovalbumin (OVA), and β lactoglobulin (BLG; Sigma Chemical Co., Poole, Dorset, England) were assayed by enzyme-linked immunosorbent assays (ELISA) previously described.⁸ Levels of IgA and IgG anti-GLI in serum were also assayed by ELISA.

For assays of Igs in jejunal fluid, serial twofold dilutions of reference preparation were used to produce a standard curve. Serial dilutions of test samples were also assayed. Results were read in an MR500 Automatic microplate reader (Biotek, Billingshurst, Sussex, England) and set at a wavelength of 405 nm (optical density [OD]⁴⁰⁵). Only when the results of at least two of the sample dilutions fell within the range of the standard curve was the assay considered statistically satisfactory and the Ig content of the sample

determined as the mean for these two sample dilutions. Human colostral IgA (Sigma Chemical Co.) was used as a standard for IgA and human reference serum (Protein Reference Unit, Sheffield, England) was used as a standard for IgM.

In the assays of specific antibodies, prior experiments were done to determine optimum test conditions for each antigen, isotype, and fluid. Serum from an untreated celiac patient, previously recognized as having high levels of antibodies of IgG, IgA, and IgM isotypes to GLI, OVA, and BLG, was used as a reference standard. The reference specimen and test specimens were studied in duplicate at a predetermined dilution, varying for the different assays, and the plates read when the OD for the standard reached 1.0. If the duplicates varied by more than 10%, the result was discarded. Antibody levels were expressed as percentages of the OD of the standard. Results are thus obtained and presented as nonparametric data; antibody levels are not directly proportional to the antigen-binding capacity of the sample. This is a feature of all such assays.

Care was taken to ensure that the results were not merely due to nonspecific binding of IgM, a matter of particular concern in assays of anti-GLI. All plates were preincubated with 1% bovine serum albumin in buffered saline with 0.5% Tween 20, pH 7.4, before addition of the samples. To confirm antigen specificity of the assays, samples of jejunal fluid were tested for IgM anti-GLI and IgM anti-OVA before and after incubation for 1 hour in GLI-coated microtiter wells. There was 50%–70% reduction of the OD reading for anti-GLI but there was no change in anti-OVA. Similar, antigen-specific reduction in activity was found after incubation in OVA-coated wells and after testing for IgM anti-OVA and IgM anti-GLI.

Classification of Antibody Data

“Normal” values for the various antibody assays were based on results in 28 immunologically normal individuals previously studied⁸; as explained above, units of measurement were OD for test specimen expressed as percentage of the OD of the standard. For serum, normal values were taken as ≤ 20 for IgA anti-GLI and ≤ 40 for IgG anti-GLI; for all IgA and IgM antibodies in jejunal fluid, values ≤ 15 were considered normal.

When jejunal fluid antibody studies showed a high value for IgM anti-GLI, together with high levels of at least two other intestinal antibodies characteristic of celiac disease (IgA anti-GLI, IgM anti-OVA, IgM anti-BLG), the specimen was designated as “celiaclike intestinal antibody” positive (CIA+) and all other specimens were considered CIA– (negative).

Counts of Intraepithelial Lymphocytes

IEL counts were performed in 94 biopsies, all with essentially normal mucosal architecture (7 celiacs on a GFD, 4 patients with DH, 44 nonceliacs from series A, 39 nonceliacs from series B). In total, there were 41 CIA+

nonceliac cases and 42 patients with a similar range of diagnoses but normal intestinal antibody patterns.

A differential count of cells was performed within the villus or surface epithelium with a Leitz microscope and $\times 100$ immersion lens (Leica, Milton Keynes, England). At least 1000 enterocytes per biopsy were counted, and the results were expressed as numbers of lymphocytes per 100 villus enterocytes.⁵

Statistical Methods

The Student's *t* test and the Mann-Whitney *U* test were used for comparing Ig and antibody levels, respectively, between groups; and the Spearman's test for comparisons of serum and jejunal fluid anti-GLI antibody levels. *P* values < 0.05 were considered significant.

Results

Series A: Diagnostic Groups and Pathology

Details of the patients, their diets, final diagnoses, and biopsy histological classification are given in Table 1.

Celiac disease and DH. Thirty-six of the biopsy samples were taken from 33 patients with celiac disease (3 patients underwent biopsy twice). There were 14 new cases of celiac disease and 22 biopsy samples from GFD-treated celiacs, the majority in the early stages of treatment (<1 year), with improved but still unequivocally abnormal histology. Five of the 6 DH patients were newly diagnosed and 1 was on a GFD.

Not celiac. There were 3 patients in whom a diagnosis of celiac disease had previously been made on inadequate criteria (no biopsy or normal biopsy); in

the final analysis they have all been classified as healthy.

Inflammatory bowel disease. Four patients had Crohn's disease and 3 had ulcerative colitis.

Idiopathic diarrhea. These 23 patients had chronic, watery diarrhea, and no firm diagnosis was made even after full investigation by a consultant gastroenterologist. One was also IgA deficient.

Oral ulceration. These were patients with severe aphthous ulceration or oral candidiasis (HIV negative) referred for malabsorption work-up by dental surgeons and in whom no evidence of intestinal disease emerged.

Miscellaneous disease. There were 23 patients with various significant organic gastrointestinal diseases including diverticular disease, small bowel bacterial colonization, colonic polyps, giardiasis, colorectal cancers, radiation enterocolitis, drug-induced diarrhea, and alcoholic cirrhosis.

Nutritional deficiencies. Seven patients had presented with anemia or weight loss ultimately attributed to poor diet.

Irritable bowel syndrome. These were patients with abdominal pain, bloating, and variable bowel habit with no identifiable organic cause.

No abnormality detected. There were a few patients who had trivial symptoms or psychiatric disease or had constitutional short stature.

Of the 98 patients with a final diagnosis other than celiac disease or DH, 93 were reported to have a histologically normal jejunal biopsy and 5 had minor histo-

Table 1. Final Classification of Patients by Diagnosis and by Jejunal Biopsy Histology

Final diagnosis	Diet	No. of patients	Age (median [range])	Histology		
				SVA/PVA	Minor changes	Normal
Celiac disease	ND	14	39 (19-82)	14		
	GFD	22	39 (14-82)	16	2	4
DH	ND	5	44 (30-60)	2	2	1
	GFD	1	20	1		
Not celiac	ND	1	34			1
	GFD	2	26 (14-38)			2
Inflammatory bowel disease	ND	7	29 (18-41)		2	5
Idiopathic diarrhea	ND	23	36 (15-67)			23
Oral ulceration	ND	6	37 (19-80)			6
Miscellaneous gastrointestinal disease	ND	23	46 (16-76)			23
Nutritional deficiencies	ND	8	49 (23-69)		1	7
Irritable bowel syndrome	ND	13	35 (20-45)		1	12
No abnormality detected	ND	15	34 (13-57)		1	14

NOTE. For details of diagnostic categories see text.
GFD, gluten-free diet; ND, normal diet; SVA, severe villus atrophy.

ical abnormalities (short villi in 2, excess inflammatory cells in 3).

Classification by Intestinal Antibody Pattern

The 140 patients were also subdivided by intestinal antibody findings into four groups: celiac/CIA+, celiac/CIA-, celiacs and DH patients who did (33 cases) or did not (9 cases) express the CIA pattern; patients with other diagnoses (nonceliac/DH) OTH/CIA+ and OTH/CIA-, patients with other diagnoses who did (16 cases) or did not (82 cases) express the CIA pattern.

Antigliadin Antibodies in Serum

Serum samples, collected at the time of jejunal biopsy, were available for 137 of the 140 cases studied. Levels of serum IgA and IgG anti-GLI antibodies are shown in Figures 1 and 2. Twelve of the 14 untreated celiacs had high levels of serum IgA anti-GLI as did 9 of the 21 celiacs on GFD. Of the 5 new DH cases, 2 had high serum IgA anti-GLI and one had high serum IgG anti-GLI. Fourteen of the 96 nonceliac, non-DH cases also had IgA anti-GLI values above 0.20, but, as illustrated, in only 1, a man with alcoholic cirrhosis, was the level particularly high. In contrast, detectable serum IgG anti-GLI was present in the majority of patients, and although a high value was found in 10 of 14 untreated celiacs, high values were also present in 10 of 96 nonceliac, non-DH cases.

Antibodies to Foods in Intestinal Secretions

The CIA pattern was present in 13 of 14 newly presenting celiacs and in 4 of the 5 new DH patients.

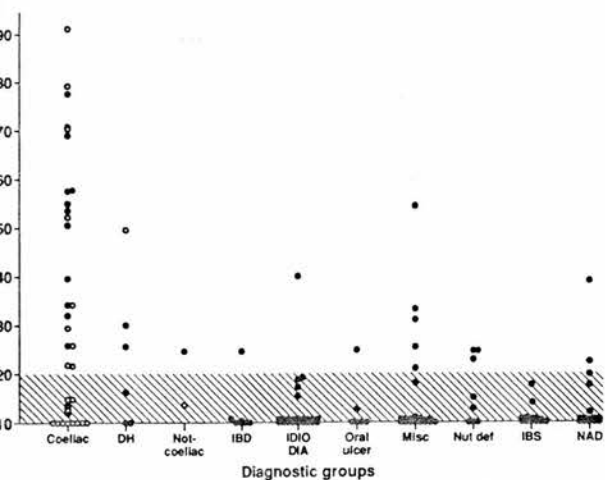


Figure 1. Serum IgA anti-glutgliadin antibody levels in patients grouped by final diagnosis (normal range is shaded). ●, ND patients; ○, GFD patients.

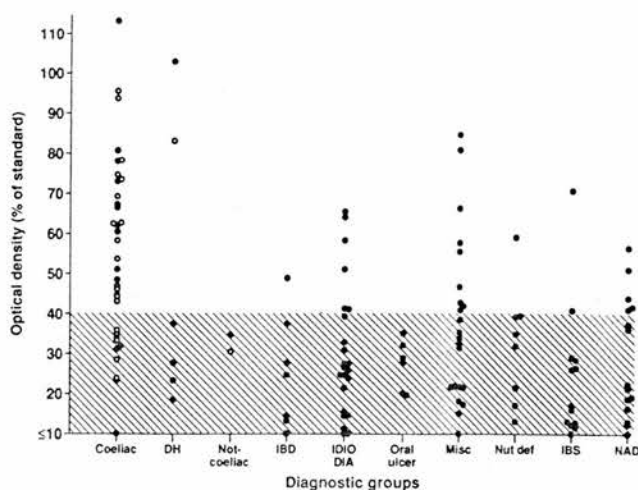


Figure 2. Serum IgG anti-glutgliadin antibody levels in patients grouped by final diagnosis (normal range is shaded). ●, ND patients; ○, GFD patients.

Clinical, biopsy, and immunological information on the two who were CIA- are presented in Table 2; there were no unusual clinical features, but it is of interest that the celiac patient, who had classical enteropathy, also had a classical celiac HLA phenotype, whereas this was not the case for the DH patient, whose jejunal mucosa was normal. Of the 22 celiacs and 1 DH patient on GFD, the frequency of the CIA+ pattern was 69.5% and 16 of the 98 nonceliac patients (16.3%) were CIA+.

Results of assays of intestinal fluid IgA and IgM anti-GLI are presented in Figures 3 and 4.

Ig Concentrations in Jejunal Fluid

As shown in Table 3, concentrations of IgA and IgM in jejunal fluid were generally higher in celiac and DH patients on a normal diet than in other diagnostic groups. When the patients were subdivided according to intestinal antibody patterns, it emerged that the two subsets with positive CIA patterns, COE/CIA+ and OTH/CIA+, had significantly higher concentrations of IgM than the CIA negative cases, irrespective of diagnosis. Total IgA concentrations were higher in the OTH/CIA+ group than in the other subsets.

Serum Anti-GLI Antibodies in Relation to Intestinal Antibody Patterns

There were no differences in the levels of serum antibodies between the CIA+ and CIA- nonceliac groups. In other words, high levels of serum anti-GLI correlated with the presence or absence of gluten-sensitive enteropathy but not with intestinal antibody levels.

Table 2. Clinical, Pathological, and Immunological Details of Two Patients (Celiac and DH) on Normal Diet Negative for the CIA Pattern

	Patient 1		Patient 2
Diagnosis	Celiac		DH
Diet	Normal	GFD	Normal (low residue)
Age	44		49
Indication for jejunal biopsy	Diarrhea		DH
	Iron-deficient anemia		Variable bowel habit, asthma, eczema
Biopsy histology	TVA		PVA
Disaccharidases			Normal
IEL count/(100 enterocytes)	35		40
			32
Lactulose/rhamnose permeability test	0.14 ^a		0.034
			0.02
Serum			
IgA-AGA	25.8 ^a		8.7
IgG-AGA	51.0 ^a		35.0
			23.1
Jejunal fluid			
T. IgA (μg/mL)	11.5		19.1
T. IgM (μg/mL)	1.4		1.5
IgA-AGA	2.2		3.9
IgM-AGA	6.1		7.8
			8.2
Other Abs	Low		Low
HLA Type	A1,11;B8,57;BW4/6		A1,2;B44,35;BW4/6
	DR3,7;DRW52/53;DQw2		DR2,4;DRW53;DQW1,3,7

NOTE. Normal values: permeability <0.04; IgA-AGA <20; IgG-AGA <40.
Abs, antibodies.
^aResults abnormal.

Clinical Details of CIA Positive Nonceliac Cases (Series A)

Details of diagnosis, reason for the biopsy, and other studies performed in the group of 16 nonceliac, non-DH cases in series A, positive for the intestinal antibody pattern, are presented in Table 4. In 6 cases the intraepithelial lymphocyte (IEL) count was high; other features of the biopsy samples were normal in 5 of these; 1 man had minor, nonspecific abnormalities with short villi. Minor abnormalities of the mucosa were also present in two cases with normal IEL counts. The sugar permeability test was abnormal in only 5 cases, including the 3 with minor histological abnormalities.

Intestinal Antibody Patterns in Series B

Jejunal fluid antibody levels were measured in an additional 119 nonceliac patients with normal jejunal biopsy histology. Positive CIA patterns were present in 2 of 8 “nonceliacs,” 2 of 14 patients with IBD, 4 of 12 with idiopathic diarrhea, 3 of 12 with mouth

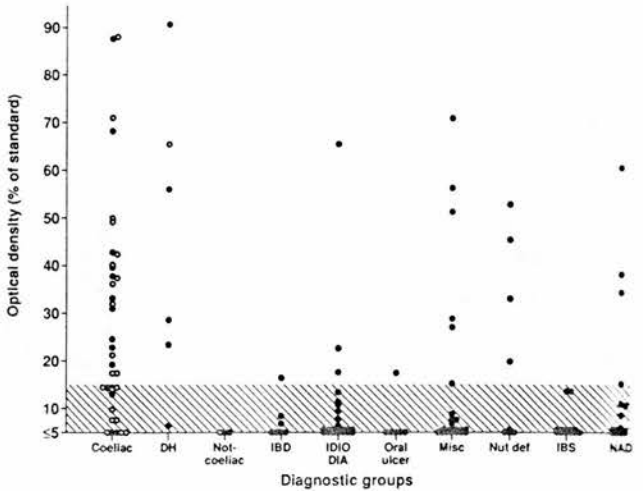


Figure 3. Jejunal fluid IgA anti-gluten antibody levels in patients grouped by final diagnosis (Normal range is shaded). ●, ND patients; ○, GFD patients.

ulcers, 7 of 34 with various other diagnoses, 3 of 7 with nutrient deficiencies, 1 of 18 with irritable bowel syndrome, and 3 of 14 with no abnormality detected.

Intraepithelial Lymphocyte Counts

With the technique used, the normal range is 10–40 IEL per 100 villus enterocytes, and virtually all untreated celiacs have abnormally high counts within the flat surface epithelium (e.g., results in our studies of four separate groups of patients have ranged from 44 to 180 IEL per 100 enterocytes).^{5,12–14} Results for biopsies from patients in series A and B are shown in Figure 5. Abnormally high counts were present in one (CIA+) of 7 GFD-treated celiacs, in 3 of the 4 DH patients (all 3 were CIA+), and in 14 patients with

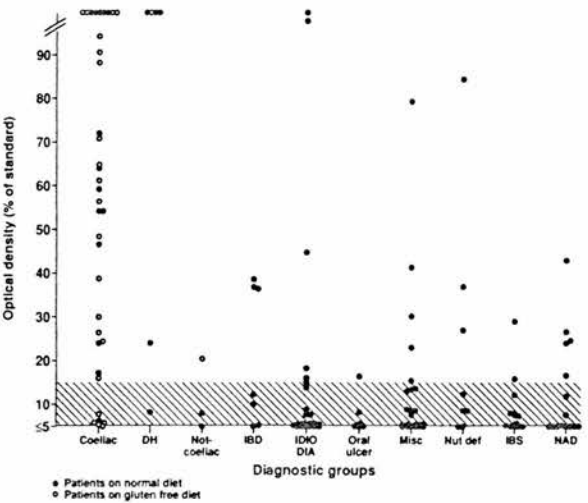


Figure 4. Jejunal fluid IgM anti-gluten antibody levels in patients grouped by final diagnosis (normal range is shaded). ●, ND patients; ○, GFD patients.

Table 3. Ig Concentrations in Jejunal Fluid

	Total IgA	Total IgM
ac		
CD	224.5 ± 244.7	25.8 ± 29.6
GFD	118.6 ± 134.4	18.8 ± 49.6
CD	223.8 ± 260	26.2 ± 30.4
GFD	132.4	14.5
celiac		
CD	39.4	8.9
GFD	193.3	16.5
Inflammatory bowel disease	213.4 ± 191.7	9.4 ± 12
Idiopathic diarrhea	153.6 ± 286.4	7.6 ± 18.9
Ulceration	43.9 ± 50.4	3.3 ± 4
Cellulaneous	137.4 ± 128.9	5.4 ± 6.9
Nutritional deficiencies	260 ± 348	24.7 ± 41.7
Irritable bowel syndrome	102.3 ± 161.9	1.9 ± 1.5
Abnormality detected	181.8 ± 206	2.9 ± 3.6
ac/DH	167.5 ± 194	21.5 ± 42
CIA+	189.3 ± 208	19.2 ± 24.6
CIA-	78.3 ± 86	2.3 ± 1.8
Final diagnosis		
CIA+	426.6 ± 335.7	25.4 ± 33
CIA-	101.6 ± 136.7	3.2 ± 4.5

NOTE: Data represent mean ± SD in µg/mL.

other diagnoses. Thirteen of these were CIA+ cases; IEL count was high in only one CIA- nonceliac patient (a woman with colonic Crohn's disease).

Clinical Effects of GFD or Gluten Loading

Eight CIA+ nonceliac, non-DH cases and one CIA- patient have had clinical trials of gluten restric-

tion. Details are presented in Table 5. Five CIA+ patients with chronic high-volume diarrhea had complete resolution of diarrhea within 1–2 weeks of starting a gluten-free (or, in one case, low-gluten) diet; in four of these, the IEL count in the initial biopsy was high. Interestingly, a GFD did not markedly influence the severe watery diarrhea of a woman with collagenous colitis (CIA-) whose identical twin is a celiac. The GFD had no effect in two CIA+ patients with inflammatory bowel disease and intractable diarrhea.

A 41-year-old CIA+ woman with recurrent severe aphthous ulceration when eating a diet with 24 g gluten daily was prescribed a GFD; she noticed some improvement but found the diet inconvenient and took it for only 5 weeks. Several months later, studies of γ/δ IEL in a stored biopsy specimen were performed and counts were found to be high. She then agreed to take 10 g additional gluten daily for a month and had another jejunal biopsy. Pathological examination revealed subtotal villus atrophy.

Discussion

Within the framework of the current definition (a permanent gluten-sensitive enteropathy), clinical, pathological, epidemiological, and immunological approaches are revealing several forms of celiac disease. In so-called active celiac disease, malabsorption and nutritional deficiencies range from profound to minimal; indeed around 50% of newly diagnosed adult ce-

Table 4. Demographic Data of Patients Positive for the Coeliaclike Antibody Pattern in Jejunal Fluid

Age	Sex	Indication for jejunal biopsy	Final diagnosis	Biopsy histology	Tissue disaccharidases	IEL count ^a	Lactulose/rhamnose permeability ratio ^b
20	F	Diarrhea	Crohn's disease	Minor changes	Not done	27	0.134
24	F	Diarrhea, abdominal pain	Ulcerative colitis Sclerosing cholangitis	Minor changes	Low (L, S)	15	0.240
67	M	Diarrhea	Idiopathic diarrhea	Normal	Normal	21	Not done
19	F	Diarrhea, weight loss	Idiopathic diarrhea	Normal	Not done	80	0.012
28	F	Diarrhea, weight loss	Idiopathic diarrhea	Normal	Normal	27	0.011
56	M	Diarrhea, IgA deficiency	Idiopathic diarrhea	Normal	Normal	35	0.033
54	F	Diarrhea	Microscopic colitis	Normal	Normal	46	0.032
43	M	Diarrhea	Colonic polyps	Normal	Normal	22	0.020
32	F	Diarrhea, anemia	Pernicious anemia	Normal	Normal	53	0.054
35	F	Anemia	Iron deficiency	Normal	Normal	43	0.022
23	M	Anemia	Iron deficiency	Normal	Normal	27	0.022
44	M	Diarrhea, fatigue	Poor diet, somatisation disorder	Minor changes	Low (L)	73	0.032
29	F	Diarrhea, abd dis/t	Irritable bowel syndrome	Normal	Not done	22	0.008
19	F	Bloating, abd dis/t	NAD	Normal	Normal	47	0.050
57	F	Bloating, abd dis/t	NAD	Normal	Not done	20	0.045
25	F	Abdominal discomfort	NAD	Normal	Normal	18	0.036

dis, abdominal distension; NAD, no abnormality detected; L, lactase; S, sucrase.

^aNormal: 10–40 per 100 enterocytes.

^bNormal: <0.04.

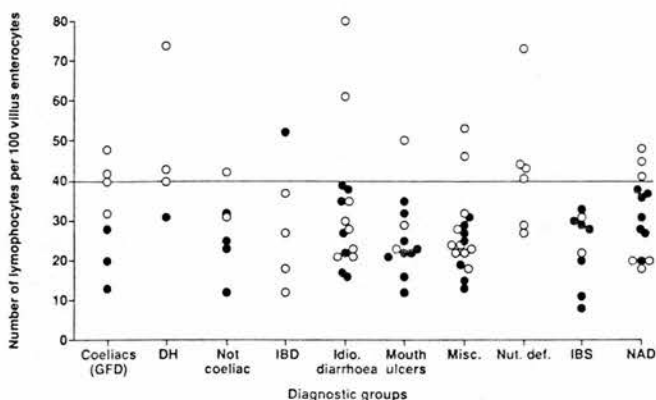


Figure 5. Intraepithelial lymphocyte counts in jejunal biopsies from 94 patients reported as normal or with minor histological changes (normal range, 10–40 lymphocytes per 100 villus enterocytes). ○, Patients positive for CIA pattern; ●, patients negative for CIA pattern.

liacs have no symptoms referable to the gut.^{15,16} Clinically silent celiac disease is being increasingly recognized (eg., in family studies where an abnormal mucosa is found in 10% of completely healthy relatives of celiacs).^{17,18} Pathologically, there is also a degree of heterogeneity. Descriptive terms such as “flat mucosa” or “total villus atrophy” are the pathologist’s shorthand for a cluster of features that together characterize the enteropathy of celiac disease. Quantitative histology¹⁹ and computerized image analysis²⁰ of biopsy samples taken during gluten withdrawal and challenge have shown that all of these features occur in a continuum with a flat, avillus lesion at one end of the spectrum and at the other end a mucosa with normal villus and crypt architecture but an abnormally high density or count of IEL. The latter would be reported as normal by most clinical pathologists.

This fact is important when one reviews the evidence for the existence of latent celiac disease and theories as to whether such patients are merely at one end of a spectrum of the pathology of enteropathy^{20–22} or whether celiac disease develops in a one- or two-stage process, latent and fully expressed.⁹ There are a few reports of normal jejunal biopsies previously being obtained from newly diagnosed celiacs.^{23–25} Morphometric analysis showed changes at the mild end of the spectrum of enteropathy in the original biopsies of two such patients,²⁵ but there are also reports of formal histological measurements showing that the original biopsy specimens were completely normal.^{23,24}

Clearly there is a need for some means other than pathological to identify the state of latent celiac disease. One approach may be by studies of IEL T cells expressing γ/δ receptors⁶; positive results have been reported in a single case detected during family studies

in Finland.²⁴ We are following another potential line, based on our observation that the intestinal antibody pattern of celiac disease also occurs in DH patients without enteropathy, and thus may provide a diagnostic test of latent celiac disease in other situations.⁹

It is generally agreed that the intestinal fluid in untreated celiac disease contains high levels of both IgA and IgM anti-GLI antibody and that, in contrast to serum antibodies, levels of intestinal antibodies remain high after healing of enteropathy in GFD-treated celiacs.^{8,26–28} In a definitive study of humoral immunity in celiac disease, we found that, in celiacs, secretions contained high levels of IgM antibodies to two other dietary proteins studied in addition to anti-GLI.⁸ Thus, we defined the CIA pattern as the presence in jejunal fluid of IgM anti-GLI together with at least two of the three other characteristic antibodies, IgA anti-GLI, IgM anti-OVA, and IgM anti-BLG.

It is now clear (Table 3) that CIA+ specimens also contain high concentrations of total IgM, in keeping with over-expression of IgM generally in the intestinal mucosa of celiacs,^{28–36} and this is not limited to food protein antibodies. In a recent investigation of intestinal immune responses to an enteric vaccine, celiacs generated high levels of intestinal IgM antibodies to a bacterial antigen, cholera toxin B subunit.³⁷

Although it was not a primary aim of the present study to assess the CIA status of celiacs, it is of interest that 1 of 14 newly presenting celiacs was CIA negative. Two of the 8 cases of DH without enteropathy previously studied by us were also CIA negative⁹ as was one DH patient without enteropathy in the present series. The fact that she lacked the characteristic HLA haplotype of celiac disease (although was DR4 positive as are other such atypical celiacs)³⁸ may be important and highlights the possibility that differences between patients in their intestinal expression of gluten sensitivity or in their mucosal immune responses may have a genetic basis. We have suggested that there may be separate genetic factors that regulate the capacity for gluten sensitization on the one hand and a susceptibility to develop severe enteropathy in the expression of mucosal T-cell-mediated immunity on the other.³⁹

We have not yet established the immunopathogenesis of the CIA pattern. Because this persists during strict gluten exclusion, the theory that it reflects a polyclonal response to lectinlike stimulation by gliadin cannot be sustained. Mucosal immunity is characterized by an overall downregulation of isotypes other than IgA.⁴⁰ The general increase in the IgM component of intestinal immunity, which we have described, could reflect either a primary disorder of B cells (i.e.,

le 5. Details of Patients Treated With Gluten-Free Diet

Symptoms and clinical diagnosis	Jejunal biopsy on normal diet		Clinical effect of gluten-free diet	Jejunal biopsy on gluten-free diet	
	Histology	IEL count ^a		Histology	IEL count ^a
Case 2: Ulcerative colitis and sclerosing cholangitis, on 10 mg prednisolone daily; developed high volume watery diarrhea after colectomy and formation of pelvic ileal pouch	Minor villus shortening	15	No effect of 6 months on gluten-free diet	Minor villous shortening	16
Case 4: Idiopathic diarrhea	Normal (on 37 g gluten daily)	80	Diarrhea resolved within 2 weeks of low gluten diet (5 g/day)	Normal	23
Case 7: Watery diarrhea. Colonoscopic biopsy showed microscopic colitis; no response to trials of prednisolone, sulphasalazine	Normal	46	Diarrhea resolved within 1 week	Normal	36
Idiopathic diarrhea, fatigue	Normal	61	Diarrhea resolved within 2 weeks	Normal	38
Intermittent watery diarrhea, iron deficiency anemia	Normal	44	Diarrhea resolved within 1 week	Not done (patient defaulted)	
Idiopathic diarrhea, iron deficiency anemia	Normal	21	Diarrhea resolved within 3 days	Normal	23
Recurrent mouth ulcers, iron deficiency anemia, no gastrointestinal symptoms	Normal	50	Improvement in mouth ulcers after 2–3 weeks	Not done (proceeded to gluten loading)	
Crohn's disease, clinically inactive but severe diarrhea after limited ileal resection (unresponsive to Cholestyramine) ^b	Normal	36	No effect of two months on gluten-free diet	Not done	
Constitutionally negative identical twin of coeliac; Collagenous colitis; severe diarrhea; poor response to trials of prednisolone, sulphasalazine.	Normal	31	Partial improvement after 6 months on GFD (stool frequency halved)	Not done	

^a >100 enterocytes.

^b Cholestyramine; Bristol-Myers, Hounslow, England.

abundance of "polyreactive" B cells, CD5+); aberrant function of the T-cell subsets that control the early stages of B cell development (ie., suppressor T-cell dysfunction^{41,42} or overactivity of local helper T cells³⁹); or the existence of an unusual immunostimulatory factor operating in the gut mucosa. Antigen-related T-cell differentiation and expansion of IgM and IgA B cells appear normal. Transepithelial immunoglobulin transport is also normal,³⁶ and indeed the production of secretory component by epithelial cells is increased in celiac disease.⁴³ An abnormally high capacity for transport of IgM and IgA across the epithelium might induce the CIA pattern. However, we have examined the molecular forms of jejunal fluid IgA (with and without secretory component) and find no differences

between CIA+ and CIA- specimens (unpublished observations). It has been suggested that IgM has much higher affinity for the secretory component than dimeric IgA. Thus, overproduction of IgM in the lamina propria may partially obstruct transepithelial IgA transport—an explanation for the presence of dimeric IgA in the serum in celiac disease.³⁹

Normal biopsy DH patients provide the only well-defined group of patients in whom in vivo dietary manipulations (the current gold standard) reveal latent gluten-sensitive enteropathy in many cases. We found that the CIA pattern was positive in 6 of 8 such patients,⁹ suggesting that non-DH latent celiacs are also very likely to be CIA positive. In the present study, 41 of 217 nonceliac patients were CIA positive; 13 of

these also had high counts of IEL and thus clearly have intestinal pathology at the mild end of the spectrum of gluten-sensitive enteropathy.^{21,22} However, in contrast to DH latent celiacs, most of these patients had symptoms suggestive of small bowel disease. Thus, quite apart from the theoretical interest in such patients, there is an important practical issue as to whether their diagnoses and management should be revised and GFD prescribed.

Although clinical effects of treatment are important to the patient, objective measures of the effects of GFD in celiac disease are mandatory to clinicians and investigators. Thus, histopathological improvement forms part of the definition of the disease. Objective assessment of a response to a GFD in the present series of patients will only be possible in those patients who have minor biopsy abnormalities. Eight of the 41 CIA+ patients (5 with high IEL count, 1 with mild enteropathy and normal IEL count, and 2 with entirely normal biopsy samples) have had a trial of gluten restriction; in 5 there was a rapid resolution of watery diarrhea and in the 4 cases in whom repeat biopsy has been performed, a high IEL count decreased to normal in 3 and there was no change in the (previously normal) IEL count in 1 case. There has been a previous report of gluten-sensitive diarrhea describing benefit from a GFD in 9 of 17 patients suffering from chronic diarrhea.⁴⁴ A CIA+ patient with aphthous ulceration took additional dietary gluten for 1 month and developed a classical celiaclike enteropathy.

The present work, together with *in vivo* gluten challenge studies in celiac patients, indicates that the expression of gluten hypersensitivity as enteropathy, measurable only if a count of IEL is performed, may be minimal. An accepted name for this type of pathology is needed, such as "high IEL mild enteropathy." This must be clearly differentiated from low-grade or other forms of nonceliac enteropathy. Further work will show how this relates to γ/δ IEL counts and whether the presence of the CIA pattern is an even more sensitive index of mucosal DTH expression than is IEL count or whether the unusual upward regulation of IgM is an independent factor, perhaps genetically determined, relevant to the induction of a state of aberrant immunity to gluten.

We suggest that some of the cases in the literature, conforming to the present definition of latent celiac disease, will, on review of biopsy pathology, be found to show high IEL mild enteropathy, thus falling within the "continuum" concept of gluten-sensitive enteropathy.²² However, there are already published and unpublished cases in whom the IEL count in the

original biopsy was normal. Not all such patients are asymptomatic; therefore the word "latent" may in any event be inappropriate in relation to the full clinical expression of gluten sensitivity. For prospective studies of candidate-latent celiacs, such as those with high IEL count, positive CIA pattern, high γ/δ expression of IEL, relatives of celiac patients, and IgA deficiency, a more generally applicable expression is needed. The term potential celiacs has recently been accepted by delegates at a workshop of the European Society for Paediatric Gastroenterology and Nutrition.⁴⁵

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INTESTINAL ANTIBODY PATTERN OF COELIAC DISEASE: ASSOCIATION WITH
 τ/δ T CELL RECEPTOR EXPRESSION BY INTRAEPITHELIAL LYMPHOCYTES,
AND OTHER INDICES OF POTENTIAL COELIAC DISEASE.

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ABSTRACT

Patients with coeliac disease have IgM antibodies and IgA anti-gliadin antibody in gut secretions, and also high counts of IEL which express the τ/δ T cell receptor. These features of intestinal immunity may be markers of latent coeliac disease. We have examined their occurrence in 77 patients referred for jejunal biopsy, in whom biopsy histology was normal, to establish to what extent these, and other candidate markers, (high total IEL count, serum IgA anti-gliadin antibody (AGA), increased permeability) co-exist in the same patient. Twelve patients had high serum AGA and 9 increased permeability. The count of τ/δ IEL was high (>5.5 per mm villus epithelium) in 9 patients, intestinal antibody pattern was positive in 21, and total IEL count high (>40 per 100 enterocytes) in 13. Overall there were 31 patients with positive indices, but in 19 only a single test was abnormal. High τ/δ IEL counts were found in 6 of the 21 intestinal antibody positive patients, but only in 2 of 56 who were intestinal antibody negative ($p<0.001$); there were no other significant associations. Clinical tests of gluten sensitivity will be required to establish the prevalence of latent gluten-sensitive enteropathy in the 40% of patients referred for jejunal biopsy in whom one or more of the immunological indices of potential coeliac disease is present.

INTRODUCTION

Coeliac disease, defined as a permanent intolerance of the small bowel mucosa to gluten, is heterogeneous in clinical presentation and pathological expression. There are also patients with "latent coeliac disease", who have a normal jejunal biopsy when eating a normal diet but at some other time, before or since, have been shown to have typical, severe gluten-sensitive enteropathy (1). Diagnostic criteria of latent coeliac disease are stringent, and the diagnosis is usually made in retrospect or by chance. Thus we have suggested that the term "potential coeliac disease" may be more appropriate in clinical practice, to be used while dietary manipulations are being undertaken in patients suspected to be latent coeliacs (2). If the findings are positive, the diagnosis of low-grade pathology coeliac disease is then confirmed.

Recent studies have revealed a number of intestinal immune features as candidate markers of latent or potential coeliac disease: high counts of T cell receptor (TCR) γ/δ intra-epithelial lymphocytes (IEL) have been shown in dermatitis herpetiformis (DH) and in a case of latent coeliac disease (3,4); a coeliac-like intestinal antibody (CIA) pattern occurs in DH patients with normal biopsy histology (5); the total count of IEL may be high as a subtle feature of enteropathy in some coeliac patients (6); anti-reticulin and antiendomysium antibodies are present in some healthy relatives of coeliac (7); intestinal permeability changes have also been reported in first degree relatives of coeliacs (8).

We recently reported that 19% of 217 non-coeliac patients referred for jejunal biopsy had a positive CIA pattern, and that a proportion of these (6 of the 9 patients who had a trial of gluten-free diet) were clinically gluten sensitive, particularly when the total IEL count was high (9). We have now undertaken a comprehensive evaluation of non-coeliac patients to establish the associations (or lack of associations) between the CIA pattern and high τ/δ IEL counts, high total IEL counts, serum IgA anti-gliadin antibody (AGA) and intestinal permeability to the probe sugars lactulose and rhamnose. We have not attempted to include systematic clinical investigations of gluten sensitivity in this series.

METHODS

Specimen collection and processing

This Gastro-Intestinal Unit provides a regional service for the clinical investigation of gastro-intestinal function. In patients referred for per-oral jejunal biopsy, the following protocol was used:

After an overnight fast, the patient swallowed a Watson biopsy capsule together with 15 mg of Metoclopramide. When the capsule had passed to the first loop of jejunum, (assessed by X-ray screening), 1-2 ml intestinal fluid were drained by gravity through the tubing. The protease inhibitor phenylmethyl sulphonyl fluoride (PMSF), 20 μ l/ml at a concentration of 0.1M, was

immediately added. The capsule was then fired to obtain a jejunal mucosal biopsy. Jejunal fluid was transferred on ice to the laboratory and stored at -70°C within 10 minutes of collection. Venous blood samples were also taken, serum separated, aliquotted and stored at -70°C . A lactulose/rhamnose test of jejunal permeability was also performed (10).

Jejunal mucosal biopsies were orientated with the aid of a dissecting microscope and divided into two pieces. One was formalin-fixed and sent to the diagnostic pathology laboratory for subjective examination by a consultant pathologist. The other piece was embedded in OCT compound, frozen, and stored at -70°C .

Reference values for CD3 and τ/δ IEL counts were obtained from results in a subset of jejunal biopsy patients, finally considered immunologically normal. Since all of these patients had been suspected to have coeliac disease, we validated this reference range by studying small bowel (duodenal) biopsies from further 26 patients, none of whom had symptoms of small bowel disease. Biopsies from the distal second part of the duodenum were collected at upper GI endoscopy, embedded in OCT compound and frozen.

Clinical diagnosis

Some of the series were coeliac patients having follow-up biopsies to assess response to GFD; a decision as to the final diagnosis for the remaining patients was made 2-3 months after the biopsy procedure, from examination of the case records, and

without knowledge of the results of immunological investigations. These non-coeliacs were grouped as follows:

Not coeliac (NOT COE) - patients in whom a diagnosis of coeliac disease had previously been made on inadequate criteria (no biopsy or normal biopsy); in the final analysis they have all been classified as healthy.

Family members (FH) - first degree relatives of coeliacs, subsequently shown to be normal.

Inflammatory bowel disease (IBD) - Crohn's disease or ulcerative colitis.

Idiopathic diarrhoea (IDIO DIA) - chronic, watery diarrhoea; no firm diagnosis made after full investigation by a consultant gastro-enterologist.

Oral ulceration (ORAL ULC) - severe aphthous ulceration (HIV negative), referred for malabsorption work-up by Dental surgeons but no intestinal disease present after full investigation.

Miscellaneous (MISC) - patients with various significant organic diseases, including small bowel bacterial colonisation, colorectal cancer, radiation entero-colitis, drug-induced diarrhoea, collagenous colitis, eczema.

Nutritional deficiencies (NUT DEF) - anaemia or weight loss, ultimately attributed to poor diet.

Irritable bowel syndrome (IBS) - symptoms of abdominal pain, bloating and variable bowel habit with no identifiable organic cause.

No abnormality detected (NAD) - a few patients with trivial symptoms, psychiatric disease, or constitutional short stature.

Duodenal biopsy patients studied

Duodenal biopsies were collected from 26 patients (17 female, 9 male; age range 16-87, mean 63.7) without small bowel disease, who had diagnostic upper GI endoscopy. Diagnoses were duodenal ulcer (4), gastric ulcer (4), hiatus hernia (3), oesophagitis (4), other GI disease (3), no abnormality detected (8).

Counts of Total Intra-Epithelial Lymphocytes

A differential count was performed of cells within the villus epithelium with a Leitz microscope and X100 immersion lens. At least 1000 enterocytes per biopsy were counted and the results were expressed as numbers of lymphocytes per 100 villus enterocytes (6). Normal values are 10-40 IEL per 100 villus enterocytes. Formal counts were performed in all 77 non-coeliac patients, and in the 5 treated coeliac biopsies with normal villous architecture.

Counts of CD3 positive and of τ/δ IEL

Serial 7 μ m cryostat sections mounted on poly-L-lysine (Sigma, Poole, Dorset) coated slides, were dried, and then fixed in fresh acetone for 30 minutes at room temperature. The staining technique was as follows. After being rehydrated in Tris buffered saline (TBS, pH 7.6) and blocked with rabbit serum (SAPU, Carlisle, Scotland) in TBS, monoclonal antibodies were then applied for 60 minutes: TCR- δ 1 (T Cell Sciences, Cambridge, MA) at dilution 1/80, or CD3 (Scottish antibody production Unit, SAPU) at dilution 1/20; followed by Biotinylated rabbit anti-mouse IgG (DAKO, High Wycombe, UK) at dilution 1/400, for 60 minutes. The sections were treated with StrepABComplex (DAKO) for 60 minutes, and the reaction was visualised using diaminobenzidine (DAB) as a substrate. Sections were counterstained with Gills number 1 haematoxylin. The appearances of a typical biopsy with a high count of τ/δ IEL are illustrated in Figure 1.

A Leitz-TAS plus computerised image analysis system (with a TASIC software operating system) was used for cell counts in frozen sections, and the results expressed as total cell count per mm of villous epithelium. Only those specimens in which at least 5 mm of epithelium could be counted, were considered technically acceptable; with duplicate counts by separate observers, mean difference in cell count was 17%, range 3-32%. Details of how the reference range was derived are presented below.

Immunoglobulin and antibody assays

In specimens of jejunal fluid, concentrations of IgA and IgM, and levels of IgA and IgM antibodies to gliadin (GLI) (gift from Dr H. Weiser), ovalbumin (OVA) and beta-lactoglobulin (BLG) (Sigma Chemical Co. Poole, Dorset, UK) were assayed by enzyme-linked immunosorbent assays (ELISA) previously described (11). Levels of IgA and IgG anti-GLI in serum were also measured by ELISA.

Classification of antibody data

"Normal" values for the various antibody assays were based on results in 28 immunologically normal individuals previously studied (11). When jejunal fluid antibody studies showed a high value for IgM anti-GLI, together with high levels of at least two other intestinal antibodies characteristic of coeliac disease, IgA anti-GLI, IgM anti-OVA, IgM anti-BLG, the specimen was designated as "coeliac-like intestinal antibody" positive (CIA+), and all other specimens were considered CIA negative (CIA-).

Sugar permeability test

Concentrations of lactulose and rhamnose in urine were assayed by High Pressure Liquid Chromatography and the percentage of lactulose and rhamnose excreted was expressed as the L/R ratio. Normal values are ≤ 0.040 .

Statistical methods

Differences in cell counts between groups were assessed by the two-tailed Mann-Whitney U test. Comparisons of frequency tables of positivity for candidate markers was by the Chi square test with Yates' correction.

RESULTS

Jejunal biopsy patients studied

Material suitable for the full range of immunological investigations was obtained from 77 non-coeliac patients (49 female, 28 male; age range 17-77, mean 37.3). Details of the patients, their diets, final diagnoses, biopsy histological classification and sugar permeability test results are given in Table 1.

As part of the technical appraisal of CD3 and τ/δ cell counts, frozen sections of jejunal biopsies from a group of coeliac patients (predicted to have high τ/δ IEL counts) was also examined. Fifteen jejunal biopsies were collected from 14 coeliac patients (11 female, 3 male; age range 16-86, mean 42.7). Thirteen were already diagnosed, attending for follow-up biopsies (one of these had two biopsies, on a gluten-free diet and during a gluten challenge); only one was a newly presenting patient.

Reference values for τ/δ IEL, and expression of results (Table 2)

A subset of 34 jejunal biopsy patients, considered at final assessment to be immunologically entirely normal, was used to establish interim reference ranges for jejunal CD3 IEL cells and τ/δ IEL counts (per mm epithelium). There were 16 with IBS, 14 with trivial or transient disorders and 4 in whom no abnormality had been detected. Reference values for the upper limit of normal (mean + 2SD) thus obtained were 67.5 CD3 IEL per mm epithelium and 5.5 τ/δ IEL per mm epithelium, respectively.

We considered whether to express results for τ/δ IEL as a percentage of the total number of CD3+ IEL, as has been the practice of some other groups. However this produced spuriously high, abnormal values in a few cases with very low total CD3 counts (presumable with deficiency of α/β IEL rather than excess of τ/δ cells). We have therefore presented data for τ/δ IEL only as the unmodified count per unit length of villus or surface epithelium, taking values >5.5 cells/mm epithelium as abnormally high.

Fourteen of the biopsies from coeliacs had abnormally high τ/δ IEL counts. The remaining patient (normal mucosa, on a gluten-free diet, with no unusual clinical features) had a normal count; his data is presented separately from the other cases.

As shown in Table 2, there were significant differences between the reference values obtained for CD3 cells in duodenal and jejunal specimens, with values for duodenum (mean 19.4 CD3+ IEL

per mm epithelium, SD 11.4) significantly lower ($p < 0.001$) than for jejunum. This may reflect a true difference between these tissues, but alternatively may be explained by the fact that patients having duodenal biopsies were significantly older than the reference jejunal biopsy group (we have previously reported low IEL counts as a feature of human immunosenescence, (12)). However γ/δ cell counts (per mm) were similar in the two sites.

There was one endoscopy patient (a woman with an uncomplicated gastric ulcer) who had a strikingly high γ/δ count at 24 per mm; in other respects her duodenal biopsy histology was entirely normal.

Comparison of CD3 and Total IEL counts

In much of the published work on latent coeliac disease, and on the spectrum of pathological expression of gluten sensitivity, IEL counts have been performed in H&E stained sections, using a conventional light microscope, with results expressed as IEL per 100 enterocytes. In the future, if frozen sections and image analysis are necessary to count γ/δ IEL, it may be preferable to count CD3+ cells per mm as an alternative to total IEL per 100 enterocytes. The material examined in the present series allows a direct comparison of these two techniques. As shown in Figure 2, there was a highly significant correlation between IEL counts in paraffin sections and CD3+ cell counts in frozen sections ($r = 0.765$, $p < 0.001$). All cases with high CD3+ cell counts had high IELs, but there were 7 cases with high IEL count, normal CD3

cell count, suggesting that a non-T subset of lymphocytes may be present in the epithelium of these patients.

Positive results for candidate markers of latent coeliac disease in 77 non-coeliac patients

These are summarised in Table 3, with patients subdivided into diagnostic groups.

The CIA pattern of jejunal fluid antibodies was present in 21 patients. There were nine patients with high counts of τ/δ IEL in the villus epithelium, and conventional IEL counts were high in 13 cases. High titres of serum IgA antigliadin antibodies were present in 12 patients, with no obvious relationship with diagnostic groups or the other markers. The sugar permeability test was abnormal in 9 cases, mainly those with mild abnormalities of jejunal pathology or with IBD.

Associations between the CIA pattern, high τ/δ counts and high total IEL counts are illustrated diagrammatically in Figure 3. High τ/δ IEL counts were found in 6 of the 21 CIA positive patients, but only in 2 of 56 CIA negative ($p < 0.001$); there were no significant associations in any other marker combinations. A high total IEL count was more frequent in CIA positive patients (5 of 21), than in patients negative for the CIA pattern (8 of 56), but the difference was not significant.

There were 31 patients with one or more positive result, but in 19 only a single test was abnormal. Of particular interest are

patients with three parameters positive - CIA pattern, τ/δ IEL count, total IEL count. Two of the three have been shown to be gluten-sensitive (9). A woman with chronic high volume diarrhoea had complete resolution of diarrhoea within 2 weeks of starting a gluten free diet, and IEL count was normal in a biopsy taken 2 months later. Another woman with recurrent severe aphthous ulceration had a trial of gluten free diet; she noticed some improvement but found the diet inconvenient and took it for only five weeks. Several months later, when studies of τ/δ IEL in a stored biopsy specimen were performed and counts were found to be high, she agreed to take 10 g additional gluten daily for a month and had another jejunal biopsy. Pathological examination revealed subtotal villus atrophy.

DISCUSSION

The existence of latent coeliac disease implies that the finding of a normal jejunal biopsy does not completely exclude gluten sensitivity. As discussed above, there is some evidence that certain minor immunological abnormalities (which persist in treated coeliac and DH patients after healing of enteropathy) may identify latent coeliacs. One of these changes, the coeliac-like intestinal antibody, or "CIA" pattern, polyclonal up-regulation of mucosal IgM responses, occurs in 20-30% of patients referred for diagnostic jejunal biopsy. We have shown that the symptoms of some CIA+ patients respond to treatment with a gluten-free diet (9).

In the present prospective study we have combined assessment of the CIA pattern with studies of another candidate marker of latent coeliac disease, a high count of τ/δ IEL, together with several other immunological and functional tests. It is important to emphasise that there is a conceptual difference between the finding of a high total IEL count and the other aspects studied. It is now generally accepted that a high density of villus IEL (e.g. as assessed by differential counts in the present study) may be the only pathological expression of gluten sensitivity in coeliac patients (13), allowing the diagnosis of a mild but significant enteropathy. However the pathophysiological, diagnostic and clinical significance of positive findings for the other parameters examined are still uncertain.

We have found a statistically significant association between a positive CIA pattern and a high density of τ/δ IELs. The CIA pattern was also correlated (but not significantly) with a high total IEL count. We have also confirmed our previous findings that patients with subtle immune changes in gut humoral immunity have entirely normal levels of serum IgA anti-gliadin antibody (9,11). A positive sugar permeability test is independent of the other features, mainly abnormal in patients with inflammatory bowel disease and alcohol- or NSAID-related problems.

Around 40% of patients referred for diagnostic jejunal biopsy, and in whom routine biopsy pathology was normal, had positive results for one or more of the tests that have been proposed as indices of latent coeliac disease. Although we have obtained evidence of clinical and mucosal gluten sensitivity in a few of

these patients (9), this has been on an opportunistic basis, unsystematic and uncontrolled. Induction of severe enteropathy by extra dietary gluten would be unequivocal proof of gluten sensitivity, but this raises ethical issues and may be clinically unacceptable to symptomatic patients. Acute enteral or rectal gluten challenge, monitored by multiple biopsies, should be more practicable. However since the histopathological effects of dietary gluten in clinically gluten-sensitive/normal jejunal biopsy patients are strikingly different from those in classical coeliacs, it is entirely possible that the pathological changes produced by acute gluten challenge in the jejunum or rectum will be equally different in latent/potential coeliacs and classical coeliacs.

With the currently available range of tests, studies of a single putative marker cannot be expected to identify all cases of latent coeliac disease. The two best indices are likely to be a positive CIA pattern and high τ/δ count. However these are not present even in all classical coeliacs. We found one of 14 untreated coeliacs and one of 6 DH patients were CIA negative (9), and one otherwise completely typical coeliac man in the present series (who also has insulin-dependent diabetes) had no τ/δ IEL. Although logistically difficult, the identification of potential coeliacs still requires studies of several candidate markers, using jejunal fluid for ELISA studies, and frozen sections of a mucosal biopsy for T cell receptor staining.

A high total IEL count, in an H&E stained section, may be due to expansion of one or more of the three main subsets of lymphocytes

within the villus epithelium of the small intestine: (i) CD3 positive (T cells) IEL which utilise the α/β T cell receptor; numbers of these rise and fall in coeliac patients with gluten ingestion and exclusion; (ii) CD3 positive (T cells) IEL which utilise the γ/δ T cell receptor; generally, counts of these cells are high in coeliac patients, irrespective of diet; (iii) a small proportion of IEL are CD3 negative cells, with no T cell receptors; their nature, in man, is uncertain. In a few patients in the present study, total IEL count was high with CD3 cell count normal, suggesting that expansion of the atypical, non-T IEL subset may occasionally occur; further patients will need to be studied in order to establish the clinical significance of this finding.

The CIA pattern and a high γ/δ IEL count occur independently in some non-coeliacs, but also co-exist more frequently than expected by chance. These phenomena may be due to separate, intrinsic, genetically determined aberrations of the constituent lymphocyte populations of the mucosal immune system, which happen to occur together in most coeliacs. Alternatively, the possession of both aberrations may increase the likelihood of full expression of enteropathy in an individual who is genetically predisposed by virtue of an independent gene for gluten-sensitivity. These aberrations may be constitutively expressed, or may be detectable only in situations of mucosal immune activation. Whatever the underlying mechanism, it is clear that the repertoire selected from the full range of potential immune cells and molecules includes intestinal IgM antibodies and γ/δ

IEL in coeliacs, whereas these are not utilised in most non-coeliacs.

We have discussed elsewhere (9) the range of possible mechanisms of the CIA pattern, including a primary defect of maturation or IgA-IgM switch of B cells, and immunoregulatory T cell dysfunction. An expanded τ/δ IEL population may be genetically determined (3,4). High counts of τ/δ IEL, but not of α/β IEL, were found in healthy relatives of coeliac patients who possessed the extended haplotype of coeliac disease (14). Alternatively, τ/δ IEL may represent an expanded population controlled by local regulatory factors, perhaps triggered by gluten (15,16). However τ/δ IEL have very limited diversity (and thus limited capacity for antigen recognition) (17) and they are not gluten-specific (18). The fact that a high density of τ/δ IELs was found in a latent coeliac patient years before the development of enteropathy (3), and our finding of high τ/δ IEL counts in several non-coeliac patients argue against their involvement in mucosal damage.

Further elucidation of the relationships between the immunological phenomena described, and the clinical entity of coeliac disease, will require not only direct, *in vivo* investigations of gluten sensitivity in potential coeliac patients, but also characterization of the genetic make-up of both coeliac and non-coeliac patients, who do or do not have the various immune abnormalities described above.

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TABLE 1. Patients studied, classified by final diagnosis and biopsy histology.

Diagnosis	(n)	Age mean (range)	Sex f/m	Diet		Histology			Permeability	
				N	GFD	N	M.Ch	PTVA	N	Abn
NOT COE	4	30.6 (17-64)	2/2	2	2	3	1		4	
FH	4	26.8 (17-44)	4/-	3	1	4			4	
IBD	6	38.5 (23-65)	3/3	6		5	1		2	4
DIA IDIO	14	32.6 (21-70)	6/8	13	1	12	2		13	1
ORAL ULC	4	27 (19-52)	3/1	4		3	1		3	1
MISC	19	42.6 (19-77)	13/6	19		18	1		17	2
NUT DEF	4	35 (30-46)	4/-	3	1	4			4	
IBS	16	38 (21-71)	10/6	16		16			15	1
NAD	6	30 (21-52)	4/2	6		6			6	
COELIAC	15*	42.7 (16-86)	12/3	2	13	5	2	8	7	8

* in 14 patients, see text

n=number studied

Age: mean (range)

N=normal

GFD=gluten-free diet

M.Ch= minor non-specific histological abnormalities

PTVA= severe partial, or total villus atrophy

TABLE 2. Reference values for cell counts in villous epithelium (mean and SD; range)

	CD3+ cells per mm		TCR γ/δ cells per mm	
Control group				
Jejunal biopsy patients (n=77)	33.7+-16.9 (6-68.7)		1.5+-2 (0-7.4)	
Duodenal biopsy patients (n=26)	19.4+-11.4 (2.3-45.9)	*	1.5+-4.7 (0-24.4)	
Excluding atypical case	18.4+-10.3 (2.3-44.1)	*	0.6+-0.6 (0-2.5)	*
Coeliac patients (n=15)	69.2+-32.9 (31.9-120)		21.5+-16.7 (5.5-66)	
Atypical case	42.2		0.2	

* p<0.001

TABLE 3. Diagnostic groups: numbers of cases positive for candidate markers of latent coeliac disease.

Final Diagnosis	(n)	CIA+ cases	high τ/δ counts	high IEL counts	Abn.L/R test	high serum AGA levels
NOT COE	4	2	1	1		
FH	4	1				1
IBD	6	2	1		4	2
DIA IDIO	14	4	3	4	1	2
ORAL ULC	4	2	1	2	1	1
MISC	19	4		2	2	3
NUT DEF	4	2	1	1		
IBS	16	3	2		1	2
NAD	6	1		3		1
COE. N diet	1	1	1	not done	1	1
Glu Ch	1	1	1	not done	1	1
GFD	13	8	12	not done	6	4

Legends for figures

Figure 1.

Serial cryostat sections of jejunum from a non-coeliac patient with high total IEL count. Immunoperoxidase staining of intra-epithelial lymphocytes with antibodies to CD3 (Figure 1a) and to TCR $\delta 1$ (Figure 1b).

Figure 2.

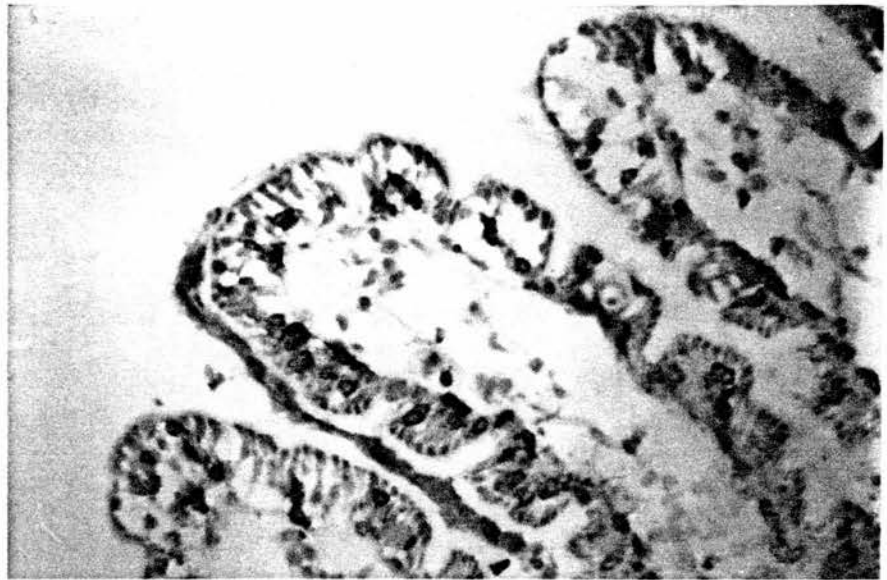
Intra-epithelial lymphocyte counts in 77 pairs of jejunal biopsy specimens. Total IEL counts in paraffin sections are expressed as IEL per 100 villus enterocytes; CD3+ cell counts in frozen sections are expressed as cells per mm length of villus epithelium.

Figure 3.

Associations between the CIA pattern, high γ/δ counts and high total IEL counts in 77 non-coeliac patients.



a)



b)

Figure 2

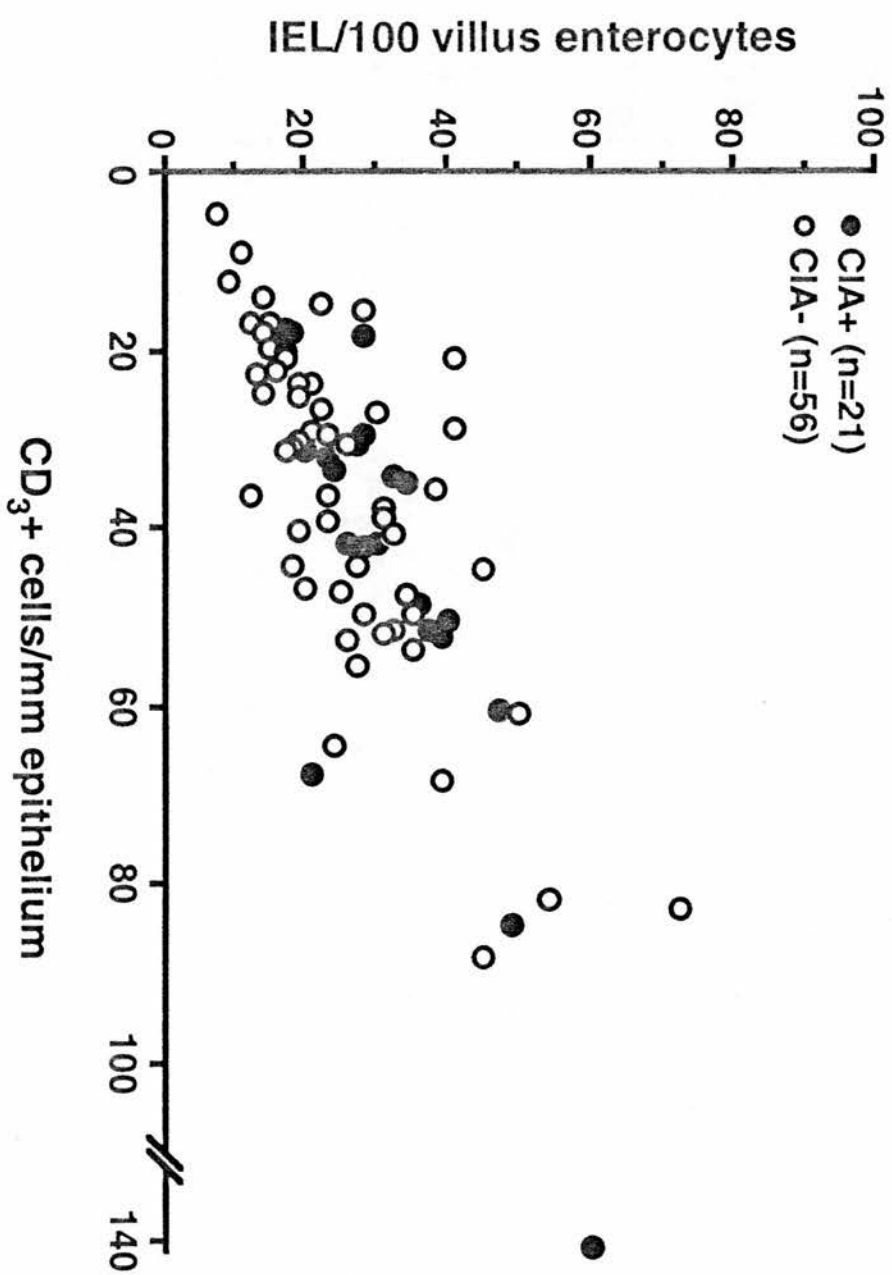


Figure 3

